Biodiscovery of Natural Products from Microbes Associated with Irish Coastal Sponges

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I, Lekha Menon Margassery, certify that this thesis is my own work and I have not obtained a degree in this university or elsewhere on the basis of the work submitted in this thesis.

Lekha Menon Margassery

Dedicated to my family

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Abstract

Marine sponges have been an abundant source of new metabolites in recent years. The symbiotic association between the bacteria and the sponge has enabled scientists to access the bacterial diversity present within the bacterial/sponge ecosystem. This study has focussed on accessing the bacterial diversity in two Irish coastal marine sponges, namely *Amphilectus fucorum* and *Eurypon major*. A novel species from the genus *Aquimarina* has been isolated from the sponge *Amphilectus fucorum*. The study has also resulted in the identification of an α–Proteobacteria, *Pseudovibrio* sp. as a potential producer of antibiotics. Thus a targeted based approach to specifically cultivate *Pseudovibrio* sp. may prove useful for the development of new metabolites from this particular genus.

Bacterial isolates from the marine sponge *Haliclona simulans* were screened for anti–fungal activity and one isolate namely *Streptomyces* sp. SM8 displayed activity against all five fungal strains tested. The strain was also tested for anti–bacterial activity and it showed activity against both against *B. subtilis* and *P. aeruginosa*. Hence a combinatorial approach involving both biochemical and genomic approaches were employed in an attempt to identify the bioactive compounds with these activities which were being produced by this strain.

Culture broths from *Streptomyces* sp. SM8 were extracted and purified by various techniques such as reverse–phase HPLC, MPLC and flash chromatography. Anti–bacterial activity was observed in a fraction which contained a hydroxylated saturated fatty acid and also another compound with a m/z 227 but further structural elucidation of these compounds proved unsuccessful. The anti–fungal fractions from SM8 were shown to contain antimycin–like compounds, with some of these compounds having different retention times from that of an antimycin standard.

A high–throughput assay was developed to screen for novel calcineurin inhibitors using yeast as a model system and three putative bacterial extracts were found to be positive using this screen. One of these extracts from SM8 was subsequently analysed using NMR and the calcineurin inhibition activity was confirmed to belong to a butenolide type compound.

A *H. simulans* metagenomic library was also screened using the novel calcineurin inhibitor high–throughput assay system and eight clones displaying putative calcineurin inhibitory activity were detected. The clone which displayed the best inhibitory activity was subsequently sequenced and following the use of other genetic based approaches it became clear that the inhibition was being caused by a hypothetical protein with similarity to a hypothetical Na^+/Ca^{2+} exchanger protein.

The *Streptomyces* sp. SM8 genome was sequenced from a fragment library using Roche 454 pyrosequencing technology to identify potential secondary metabolism clusters. The draft genome was annotated by IMG/ER using the Prodigal pipeline. The Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AMPN00000000. The genome contains genes which appear to encode for several polyketide synthases (PKS),

non–ribosomal peptide synthetases (NRPS), terpene and siderophore biosynthesis and ribosomal peptides. Transcriptional analyses led to the identification of three hybrid clusters of which one is predicted to be involved in the synthesis of antimycin, while the functions of the others are as yet unknown. Two NRPS clusters were also identified, of which one may be involved in gramicidin biosynthesis and the function of the other is unknown. A *Streptomyces* sp. SM8 NRPS *antC* gene knockout was constructed and extracts from the strain were shown to possess a mild anti–fungal activity when compared to the SM8 wild–type. Subsequent LCMS analysis of *antC* mutant extracts confirmed the absence of the antimycin in the extract proving that the observed anti–fungal activity may involve metabolite(s) other than antimycin. Anti–bacterial activity in the *antC* gene knockout strain against *P. aeruginosa* was reduced when compared to the SM8 wild–type indicating that antimycin may be contributing to the observed anti–bacterial activity in addition to the metabolite(s) already identified during the chemical analyses. This is the first report of antimycins exhibiting anti–bacterial activity against *P. aeruginosa*.

One of the hybrid clusters potentially involved in secondary metabolism in SM8 that displayed high and consistent levels of gene–expression in RNA studies was analysed in an attempt to identify the metabolite being produced by the pathway. A number of unusual features were observed following bioinformatics analysis of the gene sequence of the cluster, including a formylation domain within the NRPS cluster which may add a formyl group to the growing chain. Another unusual feature is the lack of AT domains on two of the PKS modules. Other unusual features observed in this cluster is the lack of a KR domain in module 3 of the cluster and an aminotransferase domain in module 4 for which no clear role has been hypothesised.

Objectives

1. To assess the bacterial diversity of two Irish coastal marine sponges, namely *Amphilectus fucorum* and *Eurypon major* using culture–dependent approach. By identifying the symbiotic bacteria associated with these sponges it was hoped to generate additional knowledge on the potential metabolites that they may produce. Thus by identifying novel bacteria from these sponges it was hoped that new metabolites could be discovered.
2. Screen the culturable bacteria associated with these sponge to identify potential bioactive molecules and subsequently perform chemical analysis on these molecules.
3. Employ a genome–scanning based approach on a bacterial strain which displays antibiotic activity to help identify the bioactive compounds being produced together with the potential biosynthetic pathway for these metabolites.
4. Develop and validate a high–throughput assay to identify novel calcineurin inhibitors using yeast as a model system. The aim is to use this assay to screen chemical libraries of microbial extracts, libraries of known compounds and also metagenomic libraries.

Chapter 1

Introduction

1.1 Natural Products as drugs

Chemical compounds produced by a natural or biological source such as microbes, plants or animals are scientifically termed as natural products (NP). Natural products have always played an important role in the treatment and prevention of diseases. Crude extracts from animals, plants and microbes have historically been used as medicines. This opened a new arena for the pharmaceutical industries (Ganesan, 2008), who began to investigate NP as a valuable source of therapeutics; resulting in the discovery of 877 new chemical entities between 1981 and 2002 of which 49% were NP (Koehn & Carter, 2005). This confirmed the widely held view that NP are good lead compounds for drug discovery.

1.1.1 Natural products for drug discovery

The number of new NP coming to market has declined over the last decade because the pharmaceutical companies have focused their attention on the production of synthetic molecules (Koehn & Carter, 2005). The reasons for this recent trend in the industry are both commercial and scientific. Eli Lilly and Company, one of the pioneers in the area, who started developing many β–lactam based antibiotics such as vancomycin, erythromycin, capreomycin amongst others; ceased their NP based research because of low–throughput screening and decreasing returns on their investment (Baltz, 2008).

Some of the limitations of the natural products for drug discovery are:

- The NP is often synthesized in small quantities and is required on a larger scale from a product development time line perspective. Indeed the bottleneck in drug discovery has always been the developmental process (Powledge, 2004).
- Rediscovery of known compounds.
- NP are often complex in structure.

Due to these limitations, the screening of drugs from natural products has been revolutionized to a larger extent in recent years (Lam, 2007). For a successful NP drug discovery programme the following measures have to be implemented:

1.1.1.1 Streamlining the screening process of NP

Innovative and rapid screening techniques such as automating the extract preparation and bioassay–guided fractionation is essential. Scientists from the US and China have developed an automated high–throughput system to fractionate NP from plants for drug discovery. The study has yielded multiple hits for different assays (Tu *et al.*, 2010). Separation technologies such as high–pressure liquid chromatography (HPLC), solid–phase extraction (SPE) with mass spectroscopy (MS) and nuclear magnetic resonance (NMR) are then used for the purification, separation of extracts and also detection of bioactive compound from NP. Scientists from Denmark recently developed a method called the "explorative solid–phase extraction" (E–SPE) where a solid–phase column with orthogonal selectivities is used to explore the purification strategy. Dereplication of the metabolites is facilitated by feeding the liquid chromatography–mass spectrometry (LCMS) with ionizable functional groups. This method is compatible with a wide array of bioassays and also transferable between different bioactives and organisms (Månsson *et al.*, 2010). Cubist Pharmaceuticals recently launched a program to scale–down their fermentations by using calcium–alginate macrodroplet beads in their modified fermentation vessels with an engineered *E. coli* strain which contained multiple antibiotic resistance markers to screen for the known antibiotics. This automation increased the screening process enormously and this was scalable too (Baltz, 2008).

1.1.1.2 Improvement of NP sources

For a successful drug discovery from NP the source of the compound plays an important role. Many of the compounds that have been launched on the market recently are mostly microbial in origin. Additionally, there are more in various stages of clinical trials that have anti–bacterial, anti–viral, anti–cancer and other activities. Hence narrowing the focus towards microbes as a source of novel bioactivites could prove fruitful in future drug discovery processes. A novel isolation technique would not only be helpful in the identification of novel isolates but also in the identification of novel metabolites. With the resistance developing in pathogens, there is an urgent need to develop new classes of antibiotics as many of the deaths from clinical infections are currently associated with multi–drug resistant bacteria. There is therefore a potential to curb this trend by focusing on bacteria from novel habitats (Coates *et al.*, 2011); (Kurtböke, 2010). Some of these could include marine habitats and the human microbiome amongst others (Schmitt *et al.*, 2011).

1.1.1.3 Synthetic organic methodologies

NP are indeed quite complex in structure and it is this complexity that plays a critical role in their development as drugs (Hong, 2011). Recently, many NP have been modified by total synthesises resulting in a number of FDA approved drugs that are currently available on the market. Some of the best examples include the development of the glycopeptide antibiotics oritavacin and telavancin (Van Bambeke, 2006). For example: oritavacin is an analogue of vancomycin that is currently under clinical trials (Tice, 2012).

Many industrial laboratories employ protocols involving mutation of the production strain and subsequent screens to identify production strains with a higher yield of the required product. For example scientists from Pakistan has shown an increase in the yield of alkaline protease for leather dehairing by mutating the strain *Bacillus licheniformis* N–2. The strain was then tested on goat skins and complete dehairing was observed and this coupled with the fact that the skin was also intact indicated that the mutant had not modified the collagen structure (Nadeem *et al.*, 2010).

Industries also typically employ approaches which involve the redirection of primary metabolic fluxes by the introduction of genetic modifications using recombinant DNA technologies. Some of the genetic approaches that are used for the improvement of the secondary metabolites especially produced by Actinobacteria include altering the metabolic flux, genome shuffling, overexpressing structural genes and inducing resistance to several antibiotics (Olano *et al.*, 2008). With the combination of classical and molecular genetics, many new strains of bioactive producing bacteria have been isolated and identified (Davies, 2011). The classification of the strains has also aided in defining the phylogenetic clades that helped in defining the antibiotic classes.

With the advancement of many of the aforementioned new technologies and approaches, a number of pharmaceutical industries has begun to refocus their drug discovery programmes by targeting NP (Mishra & Tiwari, 2011). Some of these companies are listed in Table 1.1.

Table 1.1 : Natural products discovered by the pharmaceutical companies and their present clinical status.

Adapted from (Sheridan, 2012).

Company	Product sources	Lead compound	Indication	Clinical status
Merlion	Microbial, fungal, plant	Finafloxacin	Infection	Phase 2
AnalytiCon	Plant, microbes	Libraries	Not applicable	Not applicable
Noscira	Marine	Tideglusib (NP–12)	CNS	Phase 2
Optimer	Microbes	Fidaxomicin	Infection	Approved

Recent reports indicate that there are around 100 NP derived compounds (Harvey, 2008) with various biopharmaceutical applications with potential use as anti–cancer agents, immunosup-

pressants, anti–infective, anti–inflammatory, neuropharmacological, vaccine adjuvants (Rey-Ladino *et al.*, 2011) amongst others. Some examples and their mode of action are listed in Table 1.2 (Vaishnav & Demain, 2011); (Chin *et al.*, 2006) .

Table 1.2 : Examples of natural products developed during these years showing their phase of development.

Drugs	Mode of action	Clinical trials	Product sources
CuracinA	Antimitotic	Pre–clinical	Marine bacterium
Platensimycin	Antibiotic	Pre–clinical	Whole cell screening
Tacrolimus	Immunosuppressant	Market	Soil sample
Dolastatin10	Anti–cancer	Phase II	Marine bacteria

A recent survey indicates that over the past thirty years that NP comprise about 41% of the newly approved drugs by the FDA (Figure 1.1). This has been possible due primarily to recent developments both in the combinatorial chemistry and the molecular biology areas (Newman & Cragg, 2012).

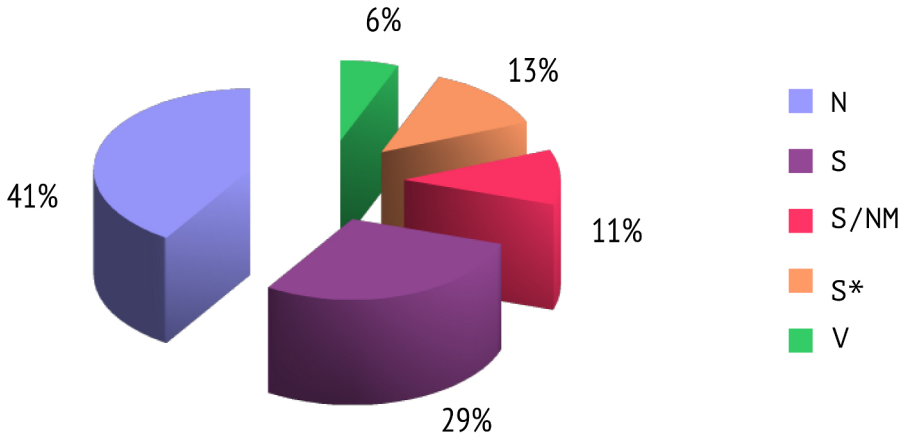

Figure 1.1 : Newly approved drugs.

N–Natural Product (biological, plant or NP derived drug), S–Synthetic drug, S/NM–Synthetic/NP-mimic, S*–Made by total synthetis/pharmacore is a NP,V–Vaccine.

Data obtained from a recent study by Newman and Cragg (Newman & Cragg, 2012).

1.2 Systematics of the Phyla Actinobacteria

Microbes play a significant role in the discovery of NP and it has been estimated that of the 23,000 currently known secondary metabolites, 42% are from Actinobacteria, 16% are from

other bacteria and the rest are from fungi (Onkarappa & Kekuda, 2010); (Kurtböke, 2012). Thus Actinobacteria are a phylum with an abundance of bioactives. Actinobacteria were initially described as being morphologically similar to a fungus–like branching filaments and so was called the "ray fungi". The different genera exhibit diversity in morphology, physiology and metabolism. Their morphologies vary from coccoid (eg., *Micrococcus*) to fragmenting hyphal forms (eg., *Nocardia*) or highly branched mycelia (eg., *Streptomyces*). Spore formation is not ubiquitous and can range from zoospores to propagules. It is evident that the observed physiological diversity may account for the production of extracellular enzymes and the numerous metabolic products that they synthesize and excrete (Gao & Gupta, 2012).

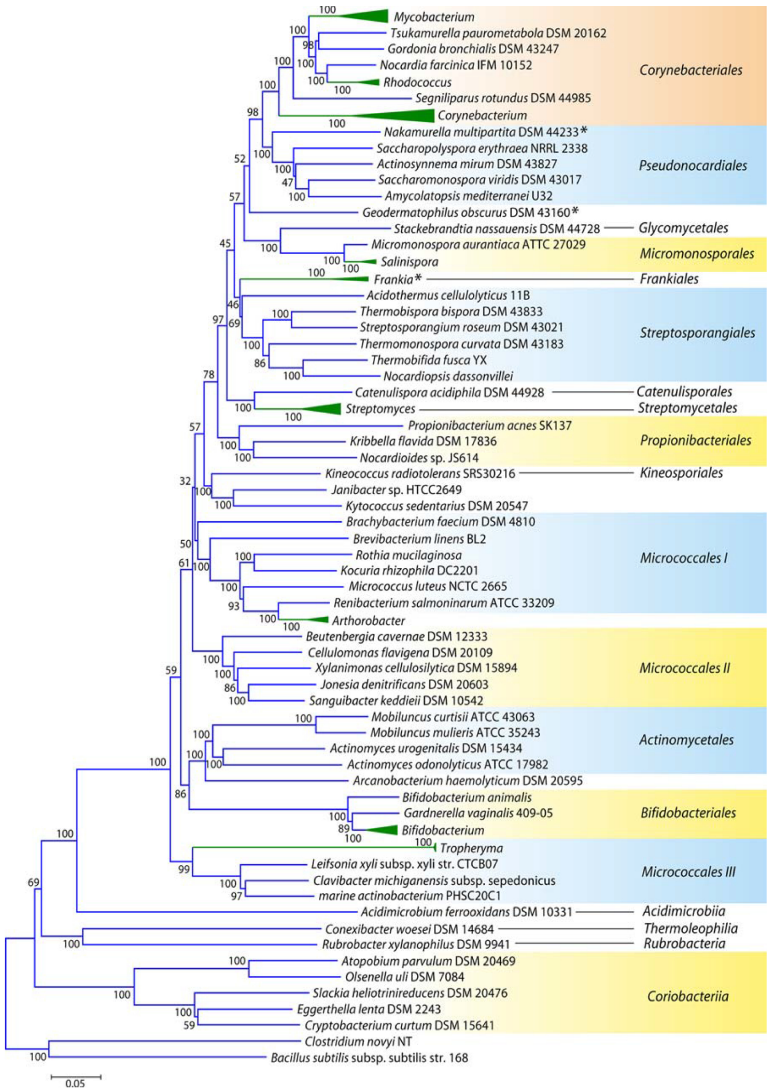

Figure 1.2 : Phylogenetic tree for 98 actinobacterial species whose genomes have been sequenced, based upon concatenated sequences for 35 conserved proteins.

The tree shown is based on neighbor–joining (NJ) analysis, and the numbers at the nodes represent the bootstrap scores of the nodes.

Adapted from (Gao & Gupta, 2012).

The members of this phyla are gram–positive with a high G+C content (>55mol% in genomic DNA), which makes this strikingly distinguishable from the rest of the bacterial community (Chater, 2006). They are free–living microbes that live in both terrestrial and marine habitat. Some of the taxonomically important features that are importance while screening for novel organisms that produce novel metabolites would be the cellular morphology, the shape and colour of the mycelium, the presence of heat–resistant spore formers and distinguishable 16S rRNA signatures (Adegboye & Babalola, 2012); (Ventura *et al.*, 2007).

Currently there are 15 suborders and according to Watve and co workers predictive modelling suggests that there may be up to 150000 bioactive molecules still to be discovered just from *Streptomyces* genus alone (Watve *et al.*, 2001). The phenotypic diversity is reflected in the genotypes (Manivasagan *et al.*, 2010); (Zhang *et al.*, 2008). Current taxonomical data suggests that there are species of at least 4 of the genera which have linear chromosomes, including *Streptomyces*, *Rhodococcus*, *Gordonibacter* and *Kineococcus*. They are distantly related to each other and this suggests that this linearization may have evolved more than once during the evolution of Actinobacteria (Figure 1.2).

1.2.1 Antibiotics produced by Actinobacteria

There are currently around 157 complete genomes which have been sequenced and 474 which are currently being sequenced from this phylum which will provide an abundant resource to data. The genomic data reveals the genomic size differs significantly within the same genus. For example, the genome of *M. smegmatis* strain MC2155 is 7.0 Mb whereas the genome of the pathogen *M. leprae* is only 3.27 Mb. However, the theory that the genome sizes may vary to facilitate adaptations to environmental changes is debatable. The molecular marker that is widely available to detect the branching patterns among different species is the 16S rRNA gene. However, there is no unique feature that is characteristic among the taxa. Hence it is essential to determine the unique properties of this phyla so that the species can be precisely defined. Studies have shown traits such as conserved indels (insertions or deletions) in protein sequences that is characteristic of the different groups of bacteria and are also helpful in understanding their relationships. The presence of novel signatures in cytochrome–c oxidase subunit I (CoxI), glutamyl–tRNA synthetase (GluRS), CTP synthetase and 23S rRNA in all Actinobacteria species are distinctive indicating that these could be useful future molecular markers (Gao & Gupta, 2005); (Gao *et al.*, 2006).

The discovery of the first *Streptomyces* antibiotic, Streptomycin in 1940s sparked much initial interest in this phylum. Since then Actinobacteria have been the source of many new antibiotics and were particularly important in the period between 1940 and 1986 which is often considered as the 'golden age of antibiotics' (Mahajan & Balachandran, 2012). A broad spectrum of compounds are produced as secondary metabolites by Actinobacteria including antibiotics, pigments, toxins, regulators and inhibitors of a signalling pathway, immuno–suppressants, immuno–modulating agents, antitumor agents, pesticides, insecticides and en-

zymes which find utility in various bioconversions (Vaishnav & Demain, 2011); (Matsui *et al.*, 2012). The major genera that produce these wide range of biological activity are *Streptomyces*, *Saccharopolyspora*, *Micromonospora*, *Amycolatopsis* and *Actinoplanes* (Onkarappa & Kekuda, 2010). A spectrum of biological applications are listed in Table 1.3.

Table 1.3 : Drugs produced by Actinobacteria.

Biological activities	Name of the drugs	Organism
Anti–bacterial	Rosaramicin	*Micromonospora*
Anti–fungal	Brasilinolide A	*Nocardia brasiliensis*
Anti–viral	AM–2604 A	*Streptomyces A*
Anti–protozoal	Coronamycin	*Streptomyces* sp.
Anti–helminthic	Ivermectin	*Streptomyces avermectinius*

It is clear that the biosynthetic pathways in this phylum have been evolving for about a billion years and the possibility exists to harness the vast metabolic capabilities of these microbes particularly targeting their ability to produce novel secondary metabolites (Baltz, 2008). This phyla has always been attractive as cell factories or bioreactors for industrial, agricultural, environmental and pharmaceutical applications. Recent advancement in the field of biotechnology have resulted in the development of a number of host–vector systems for Actinobacteria which allows for the highly reproducible production of recombinant proteins (Nakashima *et al.*, 2005). This has been possible due to two distinguishable characteristic features of this phylum:

- They exhibit a unique metabolic diversity and enzymatic capabilities.
- They have a different genetic composition (eg., high GC content) unlike the conventional host cells such as *E. coli*.

Some examples of currently available host–vector systems are shown in Table 1.4.

Table 1.4 : Expression vectors in Actinomycetes.

Adapted from (Nakashima *et al.*, 2005).

Vectors	Organism	Inducers	Location	Levels of expression
pSH19	*Streptomyces*	ϵ–caprolactum	cytoplasm	High–level
pTip	*Rhodococcus*	thiostrepton	cytoplasm	Expression at low temperature
pJAM2 Low level	*Mycobacterium*	acetomide	cytoplasm	Low level

Studies have shown that heterologous expression can provide the possibility for the production of novel bioactives (Alduina & Gallo, 2012). For example, deletion of host gene clusters in *S. coelicolor* induced overexpression of actinorhodin biosynthetic clusters in laboratory strains (Zhou *et al.*, 2012), while point mutations in the *rpoB* and *rpsL* genes are known to

result in increased levels of antibiotic production (Talà *et al.*, 2009). This indicates that gene clusters may prove to be attractive targets for modulating heterologous expression levels in Actinobacterial host systems, thereby facilitating the future development of streptomycete hosts for high–level heterologous expression of novel products.

1.2.2 Secondary metabolism

The diversity of secondary metabolism in Actinobacteria is driven by a number of different biological processes including horizontal gene transfer, point mutations, homologous recombination, gene duplications/deletions/replacements and transposition events. As previously mentioned the genome of Actinobacteria range from 3–10 MB with 5–10% of their coding capacity being devoted to Polyketide synthase (PKS) and Non Ribosmal Peptide Synthetase (NRPS) genes which are involved in secondary metabolism. In addition, recombination events involving these functional gene clusters could have resulted in the production of novel metabolites, some of which have bioactivity. One such example is the evolution of the avermectin (an anthelmintic agent) biosynthetic pathway which appears to have occurred by gene duplication and domain exchange with other PKS pathways present in *S. avermitilis* (Baltz, 2008). Genome mining of this *Streptomyces* sp. subsequently revealed the presence of 24 additional PKS and NRPS clusters, the vast majority of which are yet unidentified. Some antibiotics that involve PKS and NRPS enzymes in their biosynthesis include actinomycin, bleomycin, antibiotic TA (myxovirescin) (Figure 1.3) (Walsh, 2004).

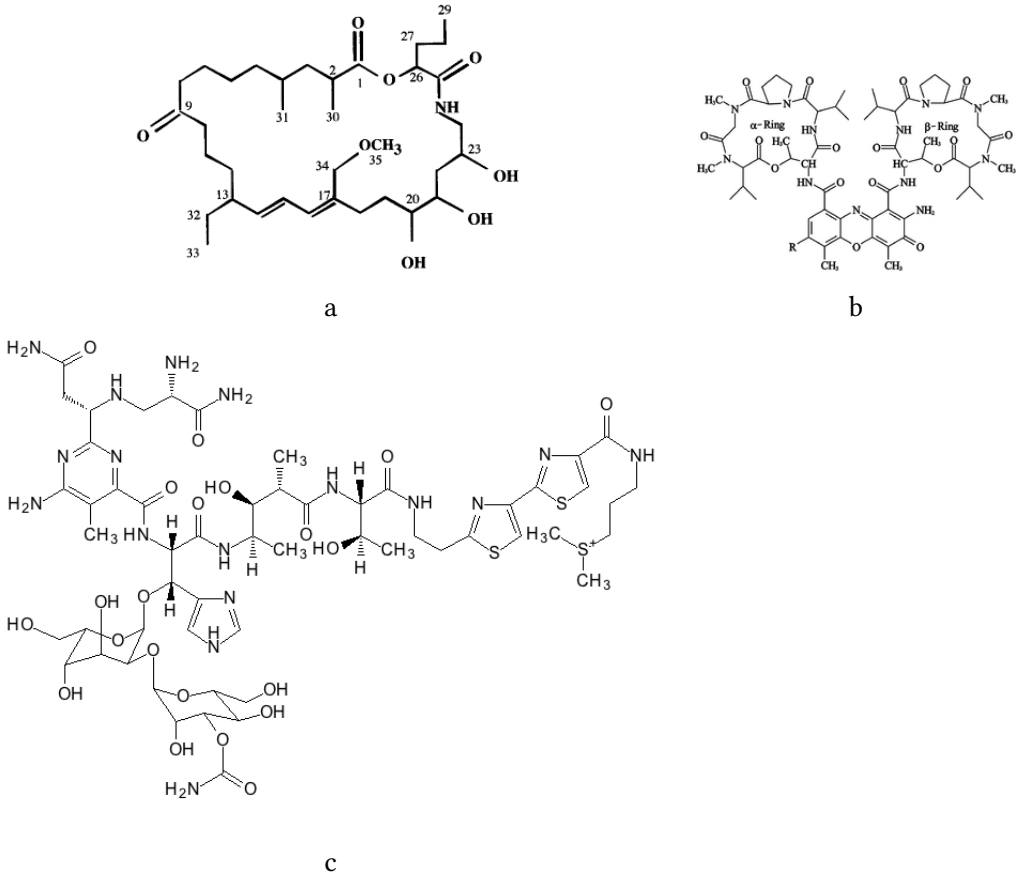

Figure 1.3 : Examples of PKS/NRPS Antibiotics.

a) PKS–antibiotic TA (anti–bacterial agent), b) NRPS–actinomycin D (anti–cancer agent), c) Hybrid–Bleomycin (anti–cancer agent). All these antibiotics are produced by the phyla Actinobacteria.

1.2.2.1 Polyketides

Polyketides are the products that are synthesised by a large family of multifunctional proteins that are involved in the production of secondary metabolites. Some examples of metabolites that originate via a polyketide pathway are erythromycin, nystatin, and doxorubicin. Polyketide architectures are quite diverse resulting in a broad spectrum of compounds such as aromatic compounds, fatty acids, macrolides and many more.

PKS are structurally and functionally similar to fatty acid synthases (FAS) as both classes catalyse the condensation of primary metabolites to form β–ketoacyl polymers that are linked to the enzyme by thioester bonds. In FAS the condensation is followed by β–ketoreduction, dehydration and enoyl reduction to yield a fully reduced fatty acid product but in PKS the reduction steps are partly or completely avoided in a controlled fashion, resulting in a highly diverse polyketide chain. PKS are categorized into three main groups, type I, type II and type III based on the enzyme structure.

Type I PKS

Type I PKS consist of multifunctional proteins with several individual functional domains and are similar to eukaryotic fatty acid synthase. The mechanism involved is described in Figure 1.4. Some of the compounds that are produced by Type I PKS include erythromycin, rapamycin, rifamycin amongst others. They are generally found in bacteria and fungi (Cox, 2007). They typically contain seven different catalytic domains namely acyltransferase (AT), acyl carrier protein (ACP), keto-synthase (KS), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), and thioesterase (TE). Type I PKS are further subdivided into iterative and modular type I PKS systems.

1. **Iterative PKS**

   This type of PKS systems, typically found in fungi, have only a single set of domains on a single polypeptide in which the active sites are reused repeatedly, as in the case of eukayotic fatty acid synthesis. The iterative PKS are divided into nonreducing PKS whose products are true polyketides such as aflatoxins (Crawford *et al.*, 2008), partially–reducing PKS such as in asperlactone (Garson & Staunton, 1981) and highly–reducing PKS, such as the lovastatin PKS (Chiang *et al.*, 2010).

2. **Modular PKS**

   Modular PKSs are the most widely studied PKS system and they possess multiple catalytic domains in which each active site is used only once. This class of PKS is commonly found in bacterial systems. The two main modular PKS are the AT-containing (*cis*) modular PKS, in which the AT domain is integrated into the modules and AT–less (*trans*) modular PKS in which the AT activity is provided by a separate enzyme. The *trans*–ATs so far identified are: free–AT, tandem ATs, tandem AT with ER and AT coupled with ER (Keatinge-Clay, 2012) . The basic steps involved in a modular PKS (*cis* and *trans*) are represented in Figure 1.4.

   The simplest functional PKS consists of a KS, an AT, an ACP and a TE domain. The domains that are responsible for the addition of a single ketide unit to the growing polyketide and the following modification are termed as modules. The modules observed in a PKS system are: a starter or a loading module that consists of AT–ACP– which is usually acetyl–CoA or malonyl–CoA, followed by elongation by repetitive decarboxylative condensations and modifications such as ketoreduction (ketoreductase (KR) domain), dehydration (dehydratase (DH) domain) and enoyl reduction (enoyl reductase (ER) domain) by the elongation or extending module –KS–AT–[DH–ER–KR]–ACP– where the elongation module is usually malonyl–CoA, methylmalonyl–CoA or ethyl–malonyl–CoA that is loaded onto the current ACP domain catalyzed by the current AT domain (the cycle is repeated for each elongation step) and finally the release or the termination module catalysed by the thioesterase domain–TE that hydrolyses the completed chain of polyketide from

the ACP domain of the previous module.

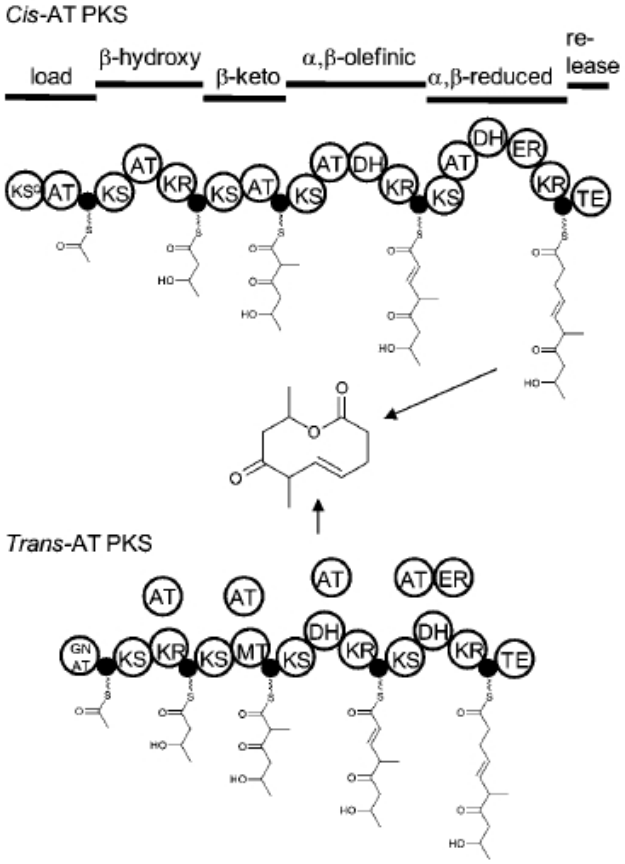

Figure 1.4 : Biosynthesis of poyketides by Type I modular PKS (*cis* and *trans*).

Adapted from (Piel, 2010).

This figure shows the biosynthesis of a hypothetical polyketide. Black filled circles represent the ACP domains. Biosynthesis is initiated when the AT selects the extender unit and loads on to the ACP domain that forms a thioester bond. Biosynthesis proceeds by the decarboxylation caused by the KS domain between the ACP and the polyketide intermediate bound to ACP domain of the preceeding module. The chain elongates by loading another ACP and the cycle continues. Some other modifications such as dehydration, ketoreduction and enoylreduction caused by DH, KR and ER domains respectively are also involved in the elongation process. All elongating modules have the core domains while the loading module lacks a KS and the last module contains an additional TE domain that releases the finished polyketide. All the AT domains in *cis* PKS systems are integrated within the modules whereas for the *trans*–AT PKS, the architecture can differ substantially from the one shown. In the figure, two *trans* ATs are represented: free AT and AT coupled with ER. An additional (optional) domain MT (methyltransferase) is observed in the *trans* AT pathway and loads a methyl group to the growing chain. KS: decarboxylating KS present in many loading modules; GNAT: acetyl-loading AT of the GCN5-related N-acetyl transferase superfamily.

Type II PKS

Type II PKS consist of an assembly of discrete enzymes analogous to bacterial type II fatty acid synthases. The chain-length factor (CLF) homologous to a KS which forms a heterodimer with the KS, the ACP and the malonyl CoA: ACP-transacylase (AT) forms the minimal PKS (Watanabe & Ebizuka, 2004). Type II PKS catalyse the formation of compounds that typically require aromatization and cyclization to complete the biosynthesis, but not extensive reduction or reduction/dehydration cycles. Type II PKS systems are involved in the biosynthesis of bacterial aromatic natural products such as actinorhodin, tetracenomycin and doxorubicin. They are mainly restricted to bacteria.

Type III PKS

Type III PKS are distinct in both structure and mechanism from the bacterial modular Type I PKS and the dissociated Type II PKS. Type III PKS catalyses the assembly of PKS by transfer of an acyl–group to the catalytic Cys, resulting in acyl-PKS complex (the starter substrate) which then undergoes decarboxylative condensations with acyl–CoA extender substrates (malonyl CoA) but without the involvement of ACPs. This is followed by Claisen condensation of the active anion with the acyl moiety to generate an acyl–CoA having an additional two-carbon unit followed by the extension of the PKS chain by repetitive reactions of the steps mentioned above. Finally, the chain elongation of a CoA-linked starter substrate is usually followed by cyclization of the linear intermediate in the same active-site cavity to generate various polyketide scaffolds (Rimando *et al.*, 2007); (Savic & Vasiljevic, 2006); (Abe & Morita, 2010). The amino residues that are important to Type III PKS and that form a catalytic triad are Cys, His, Asn. This class of PKS do not have a phosphopantetheinyl group on which the growing polyketide chains are tethered. The diverse Type III products are formed due to the specificity in the starter and the extender units, the number of incorporated extender substrates and the cyclization pattern of the intermediate (Katsuyama *et al.*, 2011). Some examples of molecules produced by Type III PKS include the synthesis chalcones and stilbenes in plants and polyhydroxy phenols in bacteria. Type III PKS systems are found in plants, bacteria and fungi.

1.2.2.2 Nonribosomal Peptides

Nonribosmal peptide derived natural products are another important family that are organised in a similar manner as the modular Type I PKS systems with multiple enzymatic domains located on a single polypeptide chain. As with PKSs the growing peptide chain is tethered to the NRPS protein via a thiol domain (T) of the peptidyl protein (Figure 1.5). Some of the pharmaceutically important drugs that are biosynthesised by this mechanism include vancomycin, bleomycin, and cyclosporin A. NRPS systems can be found in both fungi and bacteria (Rausch *et al.*, 2007).

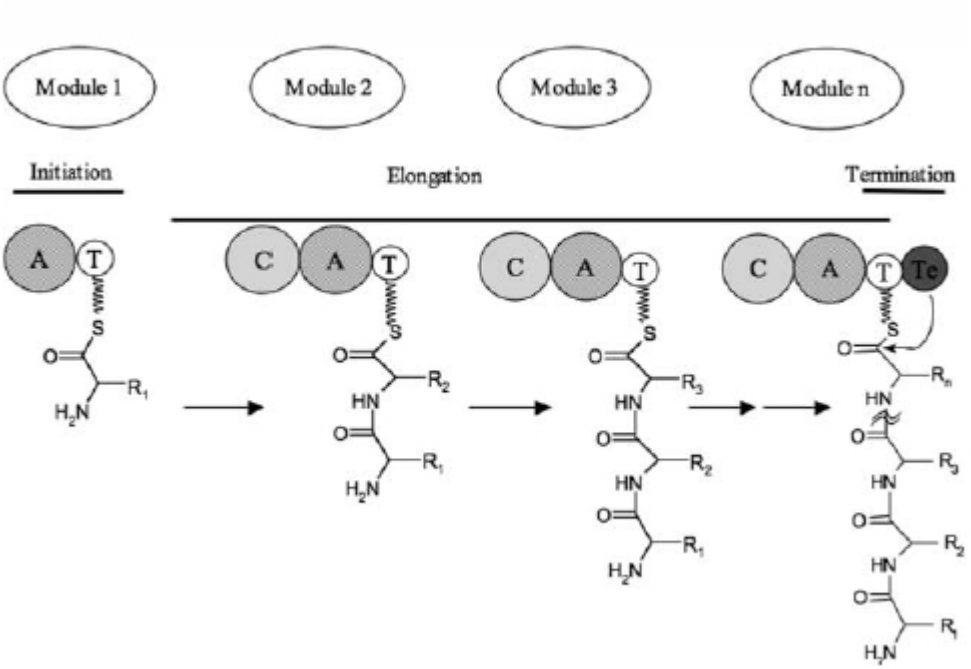

Figure 1.5 : Basic steps during synthesis of Nonribosomal peptides.

Adapted from (Donadio *et al.*, 2007).

This figure shows the biosynthesis of a hypothetical nonribosomal peptide. Biosynthesis is initiated when the A (adenylation) domain recognises and activates the amino acid and loads it onto the thiol (T) domain of the peptidyl carrier protein (PCP). The condensation domain catalyses the formation of a peptide bond amino acid and the chain elongates. The TE (thioesterase) domain terminates the process by releasing the completed peptide chain, sometimes accompanied by cyclisation.

NRPS are organised into modules that use amino acids as their building blocks to catalyse the formation of amide bonds. The order and specificities of domains within the modules determines the sequence of the final product presenting an assembly line biosynthetic system. The adenylation (A) domain is responsible for substrate selection, this domain recognises an amino acid which it activates through the formation of an aminoacyl adenylate through ATP hydrolysis and then a covalent bond is formed between the activated amino acid and the thiol (T) of the phoshopantetheinyl cofactor of the peptidyl carrier protein (PCP). The third domain is the condensation domain (C) that catalyses the formation of the peptide bonds resulting in the elongation of the peptidyl chain. Chain elongation is terminated by the thioesterase (TE) domain. This is the last module that is involved in the hydrolysis or the cyclisation of the peptide chain yielding the final product (Weber & Marahiel, 2001). Additional domains such as the epimerization (E) domain (Rausch *et al.*, 2007) for the conversion of L–amino acids to D–amino acids seen in cyclosporin A and tyrocidine A (Du & Lou, 2010), the N–methylation (NMT) domain in pyochelin biosynthesis, the heterocyclic ring formation (Cy) domain as seen in bacitracin and vibriobactin, the formylation (F) domain observed in oxazolomycin, the oxidation (Ox) domain in epothilone biosynthesis and the reduction (R) domain in pyochelin have also been identified. The final peptide products can be cyclic, linear or have a branched

structure. Once released, the products can further undergo modifications by the action of glycosyltransferases, halogenases and oxygenases (Hur *et al.*, 2012).

1.2.2.3 Siderophores

Siderophores have long been of interest to researchers due to their ability to scavenge iron. Iron is essential for the growth of all microbes and is required for electron transport pathways and for oxygen transport in higher animals. Many microorganisms produce small molecules called siderophores that have high affinity for ferric iron and they are released into the environment to scavenge iron. The siderophore–iron complex is recognized by specific receptors on the cell surface and is transported into the cell. Many siderophores function as virulence factors in pathogens and hence the enzymes that are involved in their biosynthesis are potential targets for antimicrobial therapies. Siderophores can be classified into two groups based on their biosynthetic origin: NRPS–dependent and NRPS–independent. In NRPS-dependent pathways the siderophore is produced via a NRPS system, examples include mycobactins, microcin E-492, coelichelin, and fusachelin. NRPS-independent siderophore (NIS) involve a family of synthetase enzymes that catalyses the oligomerization and macrocyclization of ω-aminocarboxylic acid that form amide bonds by several rounds as observed in desferrioxamine E. The second group involves a pair of synthetases and forms a unique hybrid NIS-NRPS pathway. This kind of mechanism is observed in petrobactin biosynthesis (Barry & Challis, 2009).

1.2.2.4 Ribosomal Peptides

Ribosomal peptide derived natural products are another class of bioactive agents that has been of recent research interest. They are usually short peptides and are post–translationally modified by various enzymes that catalyze the formation of a large number of chemical groups. The pre–peptide usually consists of a conserved leader sequence that is responsible for recognition by the modifying enzymes and the carboxyl terminus of the precursor encodes the sequence that is enzyme modified which forms the active peptide. The leader sequence is cleaved from the carboxyl terminus after modification that results in a peptide product. This kind of machinery is usually found in bacteria, fungi and in cone snails. Some of the examples are patellamides, microcin B17 and lantibiotics (Schmidt, 2010).

1.2.2.5 Terpenes

Streptomyces also produce terpene natural products that are industrially important as flavours, plant hormones and as antibiotics. The biosynthetic pathways of these have recently been identified in actinomycetes. All isoprenoid compounds, including terpenes, are derived from a five-carbon precursor called isopentyl diphosphates (IPP). Recently, it has been shown that

IPP is synthesised through the 2–C–methyl–D–erythritol 4–phosphate (MEP) pathway in most bacteria, green algae and in the chloroplasts of higher plants. Most *Streptomyces* have only the MEP pathway but some also have the mevalonate pathway (MV) for IPP biosynthesis. The first step in the MEP pathway involves the catalysation of polyprenyl diphosphate synthase (PDS) that condenses IPP into allylic diphoshates to produce linear polyprenyl diphosphates with specific chain lengths. In many cases, the polyprenyl diphosphates undergo a range of cyclizations to produce the parent skeletons–mono (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}) followed by a variety of modifications involving hydroxylation, methylation and glycosylation to produce diverse isoprenoids (Dairi, 2005). Some examples of bioactive isoprenoids include longestin (Hayashi *et al.*, 2007), which acts as a selective inhibitor of calmodulin-dependent cyclic nucleotide phosphodiesterase (Ichimura *et al.*, 1996), brasilicardin A, a potent immunosuppressive agent (Shigemori *et al.*, 1998) and pentalenolactone (You *et al.*, 2006) which is a sesquiterpenoid antibiotic.

1.2.3 Regulation of secondary metabolism

The complexity and diversity of secondary metabolism is quite a distinguishable feature of Actinobacteria. The secondary metabolite gene clusters respond to various physiological signals and stresses which mediates a wide array of signalling cascade systems. Genes involved in the production of secondary metabolites are generally arranged in clusters that vary in size from a few kilobases to 100s of kb and most of these contain specific regulatory genes for a particular pathway which are normally required for the production of these metabolites. The best known example is (p)ppGpp, a highly phosphorylated guanosine nucleotide which appears to be required for antibiotic production under nitrogen limiting conditions in *Streptomyces coelicolor* A3. However, the direct role of this ppGpp synthetase (*relA*) in antibiotic production is still unclear (Bibb, 2005).

One of the key factors that are known to contribute to antibiotic production is nutrient deprivation. Recent studies have reported that carbon catabolite control also plays an important role in morphological development thereby directly affecting antibiotic production. The utilisation of various carbon sources and exiting from the vegetative stage of growth are clearly linked. The best example is the *bldB* gene which is a DNA–binding protein only found in actinomycetes. The overproduction of this gene affects the sporulation and cell–division stage within the antibiotic producing strain as well as overall antibiotic production. In general, mutations that block sporulation seem to have a lesser effect on antibiotic production than known *bld* mutations. The reason for this is that the switch to secondary metabolism is closely connected to the early stages of morphological differentiation (Wezel & McDowall, 2011).

Additional physiological triggers/factors which are also known to influence the production of secondary metabolites in Actinobacteria include pH, temperature and oxygenation. These factors have been employed in fermentation based approaches to increase the yield of different secondary metabolites and coupled novel screening strategies have played an important role

in the isolation and development of new metabolites (Olano *et al.*, 2008).

1.3 Screening strategies

Since the discovery of drugs such as penicillin and streptomycin, many NP have been introduced to the market by industry but the number of new drugs which have discovered has fallen markedly since the 1990s. As previously mentioned there has been a dramatic increase in the emergence of drug–resistant pathogens and hence the identification of new classes of antibiotics is now more important than ever before. Since 1990 the pharmaceutical industry has only introduced three new classes of antibiotics to the market, namely oxazolidinones, lipopeptides and mutilins (Figure 1.6) (Fischbach & Walsh, 2009); (Coates & Hu, 2007); (Livermore *et al.*, 2011).

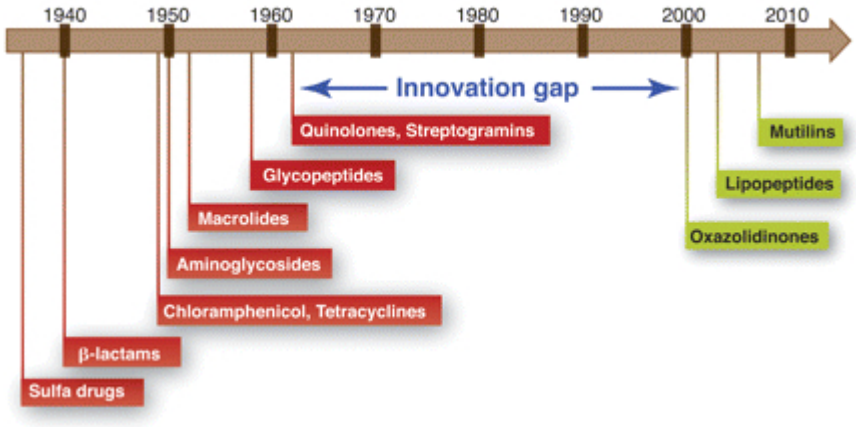

Figure 1.6 : Antibiotics discovered between 1930 and 2010.

First classes of antibiotics discovered between 1930s and 40s but after 1960s, no new classes were discovered until 2000. New screening strategies revolutionized the drug discovery.

Adapted from (Fischbach & Walsh, 2009).

The strategies that can be employed to identify novel bioactives mainly focus on the compounds, nucleotide sequences and the uncultured microbial communities (Figure 1.7).

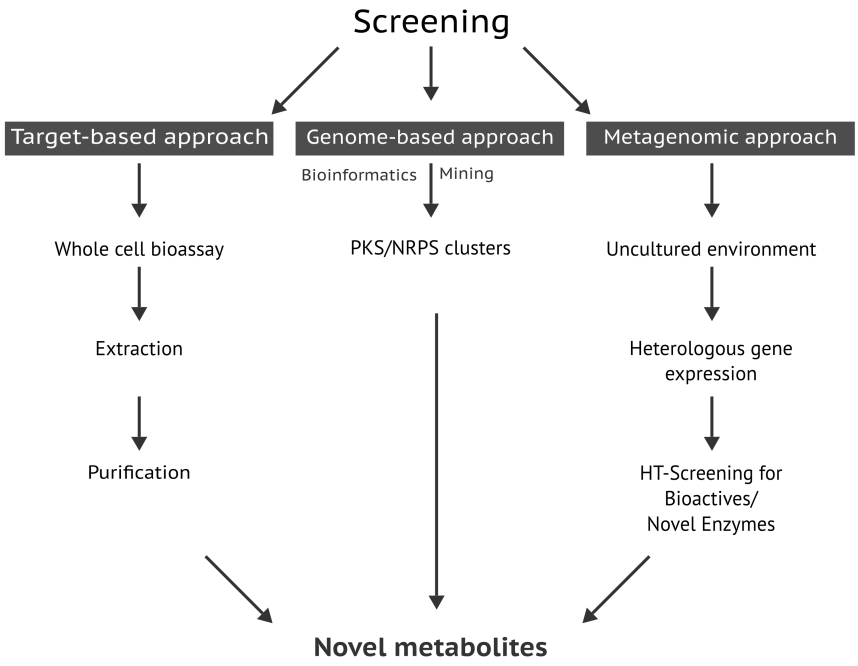

Figure 1.7 : Screening strategies in drug discovery.

1.3.1 Target–based approach

A target–based approach is the most efficient and least ambiguous method in isolating an active molecule for the target that is responsible for a pathological process or a disease that is thought to be a single gene product or group of products in a system. This approach is often referred to as the "reductionist" approach (Kenakin, 2009). The first step in this approach is mainly the identification of molecular targets followed by specific chemical– or antibody based small molecule modulators or inhibitors of the target. Theoretically, a single molecular mechanism is sufficient to have a therapeutic effect however it has to date been ineffective in treating cancer and diseases affecting several multiple cell types (Aggarwal *et al.*, 2007). The conventional phases of drug discovery can be divided into three stages: the first being the discovery phase that involves the identification of a valid target, the second being the lead optimization phase where the molecules are synthesised and tested and the third phase is the clinical development phase (Figure 1.8). In terms of strategies for drug development, the latter two steps are common however, the target validation step is unique to target–based drug discovery.

Figure 1.8 : Target–based drug discovery.

This approach involves novel targets and once validated it moves to the next process of the drug development until it reaches the market.

Adapted from (Cong *et al.*, 2012).

The concept of cell–based assays is that because of the high–throughput strategies that have been developed, thousands of compounds can be screened in a day. There is always however a possibility of missing promising drug candidates (Crowther *et al.*, 2012). The reporter assays can also lead to the false identification of the molecules. For example, in G–protein coupled receptor (GPCR) assays, fusion proteins are used. Preliminary screening of a compound which shows an effect on GPCR activation may be subsequently hard to confirm if the activation is due to the effect of the compound on the protein rather than the actual protein itself (Kotz, 2012).

Some of these limitations can be overcome by modifying the existing methods. The best example is the ADME/Tox assays. This system involves the examination of the effects of an organism, tissue or cell on a compound as well as the effect the compound has on an organism, cell or tissue (Larson *et al.*, 2011). The introduction of Mass–Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) Imaging has helped scientists to determine the mass and also elucidate the structure of the drug respectively (Capon, 2010). MS based assays can detect direct effects of drugs without manipulating cells and also quantitatively identify true changes in the metabolites. Techniques such as fragment–based drug design provides leads for previously known targets and works only on small soluble targets and has been successful in anti–infective research. The three–dimensional structure of the binding mode of the fragments is determined by X–ray crystallography or NMR to determine the properties of the drugs (Murray & Rees, 2009); (Sun *et al.*, 2011).

The chemical genetics–based target identification represents a powerful and a more recent approach that involves the identification of a novel and validated target in a more efficient and unbiased manner. With this approach, the biological activities and a target potential is assessed earlier in the discovery process which improves the efficiency of the drug discovery. Exploring new approaches such as using various biochemical and bioinformatics tools may provide possible solutions in the discovery of new metabolites.

1.3.2 Genomic analysis of Streptomycetes

As previously mentioned the genus *Streptomyces* is of interest because of its ability to produce numerous bioactives that have found utility in human and veterinary medicine and agricultural based applications. The era of genome sequencing has resulted in the identification of gene clusters which not only encode novel antibiotics but also other small bioactive

molecules; and this has opened up a new strategy in the field of drug discovery. The first antibiotic–producing actinomycete genomes to be sequenced were initially reported in the early 21st century from *Streptomyces coelicolor* and *Streptomyces avermitilis* (Challis & Ravel, 2000). Both these genomes were found to contain an abundance of genes that encoded for enzymes involved in secondary metabolism with numerous NRPS and PKS being present. The genome analysis of *S. coelicolor* revealed the presence of two dozen clusters encoding for pigments, complex lipids, signalling molecules and iron–scavenging siderophores (Borodina *et al.*, 2005) and 30 clusters from *S. avermitilis* (Ikeda *et al.*, 2003); (Omura *et al.*, 2001). Remarkably, nearly all the clusters from both the genomes probably encode different compounds in the two species.

Gradually, many other genus of this phyla were sequenced and many novel clusters were subsequently identified. A high–throughput genome scanning was undertaken by Ecopia Biosciences wherein they identified several gene clusters and predicted the structures which were likely to be produced by these clusters using the signature PKS/NRPS sequences (Challis, 2008). They grew these actinomycetes in different media and found a novel anti–fungal agent being produced by *Streptomyces aizunensis* and a novel anti–bacterial agent by *Amycolatopsis orientalis* (McAlpine *et al.*, 2005). Seven complete genomes of Actinobacteria are now available: two *Mycobacterium tuberculosis* strains, H37Rv (Cole *et al.*, 1998) and CDC1551 (Fleischmann *et al.*, 2002); *Mycobacterium leprae* (Cole *et al.*, 2001), *Corynebacterium diphtheriae*, *Corynebacterium glutamicum*, *S. coelicolor* (Bentley *et al.*, 2002) and *S. avermitilis*. In addition there are around 60 genomes which have been submitted to the NCBI from the *Streptomyces* genus alone. This provides scientists with the opportunity to exploit these genomes by decoding the metabolic pathways and to potentially identify novel metabolites that does not possess any conserved PKS/NRPS signatures (Davies, 2011).

Genomic based studies can also provide a possible route whereby variants of already existing drugs can be produced by the construction of large genomic libraries with various PKS, NRPS and hybrid clusters (rapamycin, FK506 for example) (Bode & Müller, 2005). These types of genetic alterations coupled with the feeding in of different monomers to actinobacterial cultures can thus lead to novel natural–product–derived variants (Clardy & Walsh, 2004). Studies by scientists in Japan have shown the presence of at least 25 secondary metabolite clusters in *S. avermitilis* which displayed homology to polypeptides of known function. Another example is of the proteasome inhibitor, salinosporamide A from *Salinospora tropica* where the biosynthetic gene cluster was identified for the compound in the absence of the compound being observed following analyses of fermentation broths (Udwary *et al.*, 2007). Recent studies in a *Streptomyces* sp. strain S4 identified multiple potential antibiotic biosynthetic pathways and several novel clusters that may lead to the potential novel bioactives (Seipke *et al.*, 2011). Halometabolites have recently been identified in a variety of marine microorganisms following *in silico* mining of genomes (Zhao, 2011). In addition screening for genes that encode targeted enzymes such as halogenase in the biosynthetic pathway has identified some novel putative genes.

Other genome mining based approaches have also resulted in the identification of several cryptic gene clusters that may produce novel bioactives. The strategies employed have either involved induction of expression in the natural host or heterologous expression in the surrogate strain. The first approach involves the use of different media and/or if possible, the genetic manipulation of the strain. The second involves the heterologous expression of clusters in surrogate hosts (Gomez-Escribano & Bibb, 2011). Expression of an identified pathway in a heterologous host has been successfully employed with known compounds such as the macrolides. This approach not only helped in the identification of the gene clusters but also facilitated their cloning into cosmid or BAC vectors and the subsequent transfer to a strain which grows at a faster rate than the parent strain or is known to produce larger levels of the secondary metabolites (Penn *et al.*, 2009). The popular heterologous expression host *E. coli* has never been a good host for the expression of actinomycetes gene clusters. For example, studies have reported enhanced heterologous expression of two *Streptomyces griseolus* cytochrome P450s and *Streptomyces coelicolor* ferredoxin reductase enzymes with hydroxylation activity in *S. lividans*; while these activities were not observed when *E. coli* was used as a surrogate host (Hussain & Ward, 2003). *S. coelicolor and S. lividans* has been widely used as heterologous hosts (Alduina & Gallo, 2012); (Jones *et al.*, 2012). Many new constructs for effective expression of gene clusters in heterologous hosts has been improved by removing competing pathways (Davies, 2011); (Komatsu *et al.*, 2010). All the above examples of combinatorial biosynthesis indicate that the PKS and NRPS pathways can be applied to produce novel and highly active compounds (Baltz, 2008). Structural scaffolds and docking on to the protein targets with a combination of chemical modification has proven successful in some studies (Izumizono *et al.*, 2011).

With the exponential increase of genome sequencing and genome mining analyses, many NP are now becoming therapeutic agents. The divergence from the pure chemical analyses to a combinatorial approach of both chemical and genomic approaches is the future for efficient drug discovery. These examples prove that genome–based studies can be useful in the identification of novel NP (Kurtböke, 2012).

1.3.3 Metagenomic approach

This is a term which is used to describe the study of entire communities of microbial organisms using shotgun sequencing technology rather than the clonal culture of individual strains (Handelsman *et al.*, 1998). It generally implies mixed samples of genomes from multiple species. Some other terms that are often used to describe this approach are environmental genomics, ecogenomics, community genomics and megagenomics (Chistoserdova, 2010). The approach mainly bypasses the problems of bacterial sampling and culturing and hence it is commonly referred to as a culture–independent approach (Juengst & Huss, 2009). The initial shotgun sequencing projects involving metagenomic DNA from seawater and soil samples provided insights into the enormous levels of microbial biodiversity present within these

ecosystems as well as their huge biotechnological potential particularly as a "treasure trove" of genes with not only potential novel functionalities but also of new genes that were only remotely similar to existing genes with known function (Venter *et al.*, 2004); (Tringe *et al.*, 2005); (Tyson *et al.*, 2004).

The construction of metagenomic libraries has proven to be a powerful tool for exploring the diversity of microbes in an uncultured system and thereby forming the basis of genomic studies linking the phylogenetic and functional relationships between microbes and their environment (Singh *et al.*, 2009). The technique involves isolating the genomic DNA of approximately 40–200 kb in size from an environment, and subsequently cloning it into a suitable large cloning vector such as a cosmid, fosmid or BAC based system and transformation into a heterologous cultured organism that can be used for further analysis and long–term preservation (Figure 1.9). *E. coli* is typically used as a heterologous host strain but due to its somewhat limited ability to express DNA, a number of other host strains including *Streptomyces lividans*, *Rhizobium leguminosarum*, *Pseudomonas aeruginosa*, and *Ralstonia metallidurans* together with additional shuttle vectors are beginning to be increasingly used for the detection of both secondary metabolites and novel bioactive compounds (Courtois *et al.*, 2003); (Wexler & Johnston, 2010). These clones can be used for phylogenetic analyses or screening for enzymatic activity (Walter *et al.*, 2005); (Selvin *et al.*, 2012) or for antibiotic production (Md. *et al.*, 2009). A significant problem that can occur during the construction of a metagenomic library is that fragmentation of the DNA can occur thereby causing a significant loss of the total gene complement. To overcome the fragmentation, an alternative approach is to employ blunt–end or T–A ligation to clone randomly sheared metagenomic fragments (Wilkinson *et al.*, 2002).

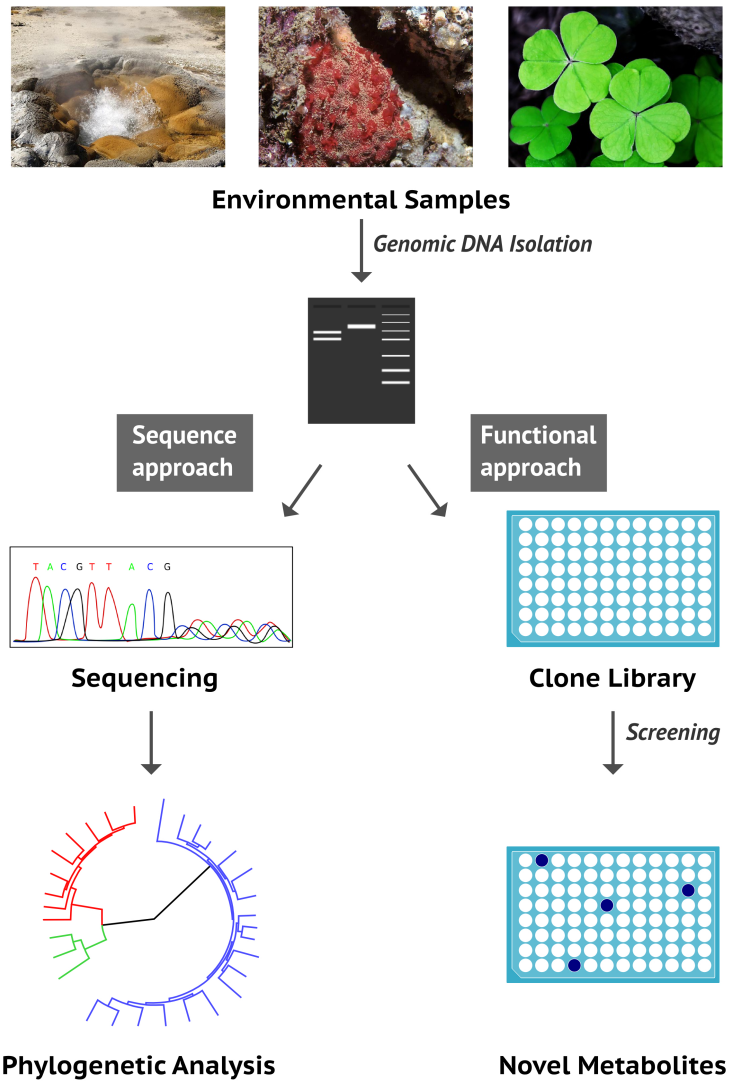

Figure 1.9 : Metagenomic Mining for Novel enzymes.

DNA is extracted from the environment samples and then it is subjected to either sequence–based or function–based approaches or both. This would help in the speciation of novel microbes or in the identification of novel bioactives.

Several bioinformatics based tools and databases have been developed to help process the large datasets being generated from the large number of metagenomic studies currently being undertaken. These methodologies have largely focused on attempts to capture the as yet unexplored microbial diversity as well as overcoming the existing barriers in estimation of diversity. In addition new screening methods continue to be developed to help select for specific functional genes within metagenomic libraries.

To analyse the metagenomic data, two approaches which are routinely employed to analyse metagenomic derived data, namely sequence–based and function–based approaches will now be discussed.

- sequence–based approach
- function–based approach

1.3.3.1 Sequence–based approach

Sequence based approaches involves targeting the presence of specific genes encoding proteins of interest being targeted by using either PCR primers or hybridization probes for these genes. Since this approach involves the design of primers or DNA probes which are derived from conserved regions of already well characterized genes or protein families, thus these approaches typically lead to the identification of new variants of known classes of proteins; and usually result in very few really novel genes being detected. Notwithstanding this however, such homology based strategy have led to the identification of genes encoding a variety of novel enzymes. Using the homology based approach, PCR amplification is performed following which the putative gene is sequenced and then subsequently cloned into an appropriate expression vector where the heterologously expressed protein is then purified and biochemically characterized to ascertain its function.

From a marine bioactive standpoint the power of data generated from the sequencing of metagenomic DNA from marine environments is clearly demonstrated in the recent report from the Sherman group (Rath *et al.*, 2011). This group sequenced DNA from the microbial symbiont community associated with the tunicate *Ecteinascidia turbinata* which resulted in the identification of twenty five genes which were then found to comprise the core Non Ribosomal Peptide Synthase (NRPS) biosynthetic pathway cluster which is involved in the synthesis of the anti–cancer agent ET–743 (Yondelis). The group subsequently demonstrated that the γ–proteobacterium *Candidatis Endoecteinascidia frumentensis* a symbiont of the tunicate produces the tetrahydroisoquinoline core of the ET–743 molecule (Rath *et al.*, 2011).

Exploration and analysis of the microbial diversity from environmental samples is termed as "taxonomical binning". The approach can be based on sequence similarities, sequence composition or both. The binning methodology depends on various factors such as the complexity of the microbial community, available reference sequences and resources (Mande *et al.*, 2012). The initial steps involve the assembly of the reads into consensus sequences called contigs which is then followed by the prediction of the genes. There are many web servers such as MG–RAST (`http://metagenomics.anl.gov/`), IMG–M (`https://img.jgi.doe.gov/m/`) or CAMERA (`http://camera.calit2.net/`) that offer computational resources (Dröge & McHardy, 2012) for this type of metagenomic analysis (Mande *et al.*, 2012). MetaProdigal, a metagenomic version of the gene prediction program Prodigal that can identify genes in short, anonymous coding sequences with a high degree of accuracy was recently launched to overcome the limitations of the current sequencing technologies. The novel method consists of enhanced translation initiation site identification, an ability to identify sequences that use alternate genetic codes and confidence values for each gene call. The program can analyse frag-

ments independently as it utilizes multiple processors (Hyatt *et al.*, 2012). Once the sequence data has been generated then it can be analysed for taxonomical classification using RDPII (Cole *et al.*, 2003), greengenes (`http://greengenes.lbl.gov/cgi-bin/nph-index.cgi`) or ARB–SILVA (`http://www.arb-silva.de/`). The advent of metagenomic approaches not only provides information about the uncultured bacteria but also about their metabolic potential (Heidelberg *et al.*, 2010).

1.3.3.2 Function–based approach

Function–based approaches involve the screening of clones for a specific activity resulting from the expression of a particular function by the clone, followed by the subsequent sequencing of the clone to identify the gene(s) involved. These screens are based on the activity being exhibited by the metagenomic clones resulting in the generation of a readily identifiable phenotypic change. For example metagenomic clones displaying lipolytic activity can be observed as zones of clearing on LB agar supplemented with 1% tributyrin. This approach has successfully been employed to identify a variety of different genes and proteins including proteins that inactivate antibiotics, genes conferring resistance, $Na^+(Li^+)/H^+$ transporters (Majerník *et al.*, 2001) and genes encoding multidrug efflux pumps that plays a major role in the development of drug–resistant microbes (Schmieder & Edwards, 2012). Another approach involves the induction of gene–expression for the identification of novel catabolite genes. Some of the function–based approaches are listed in Table 1.5.

Table 1.5 : Examples of enzymes identified by functional metagenomic approaches.

Adapted from (Simon & Daniel, 2009).

Target	Source	Sampling site	Screening approach
Lipase	Fosmids	Baltic sea sediment	Phenotypical detection
Lipase/Esterase	Plasmids	Soil from a meadow	Phenotypical detection
Cellulase	Phagemids	Crater Lake enrichments	Phenotypical detection
Antibiotics	Cosmids	Tank Water	Phenotypical detection

One of the major problems with functional based screening approaches is that expression of genes in heterologous hosts is often sub optimal. This lack of efficient transcription of genes derived from the metagenome coupled with poor secretion of the foreign protein, a misfolding of the desired protein owing to a lack of essential chaperones, a lack of cofactor synthesis or insertion into the recombinant metagenomic protein and a different codon usage of the expression host strain can lead to a low frequency of clones displaying the desired activities. Several studies has recently addressed some of these problems by constructing novel vectors, novel host strains and transposable promoters (Troeschel *et al.*, 2010), thereby facilitating more efficient transcription of metagenomic DNA.

Coupled with the development of approaches to increase the efficiency of gene transcription,

there have also been advances in the development of various high throughput screening methods such as the metabolite–related expression (METREX) system in which the activity sensor is in the same cell as the metagenomic DNA. This method has resulted in the identification of clones which produce a quorum–sensing inducer for expression of green fluorescent protein (GFP) (Tyson *et al.*, 2004). Another intracellular screening system which has been successfully developed is substrate–induced gene expression screening (SIGEX) for the isolation of catabolite genes (Uchiyama *et al.*, 2005). This system is based on the expression which is generally induced by relevant substrates, or controlled by promoters present near the catabolic genes. The construction of an operon–trap GFP–expression vector available for shotgun cloning that allows the selection of positive clones in liquid cultures by fluorescence–activated cell sorting made SIGEX a high–throughput screening method. This approach has been implemented in the cloning of aromatic hydrocarbon–induced genes from a groundwater metagenome library and subsequent genome–informatics analysis was performed (Uchiyama & Miyazaki, 2010). The use of an engineered host strain to detect particular cellular pathways using reporter gene strategy coupled with high–throughput assays has recently proven successful. For example scientists from France employed a high–throughput assay using a reporter–gene based strategy to allow rapid detection of NF–κB modulation to screen a metagenomic library from the human intestinal microbiota of patients suffering from Crohn's disease, allowing the identification of NF–κB modulating clones. They employed a human colorectal cell line HT–29 containing the secreted alkaline phosphatase (SEAP) reporter gene and identified excellent candidate metagenomic clones that can be used to further study novel host–commensal interaction mechanisms (Lakhdari *et al.*, 2010).

1.4 The Marine Environment as a source of Natural Products

As previously mentioned NP have historically been a rich source for drug development For example from 1981 to 2002, of the 1031 new candidate drugs approved by the FDA; 23% were natural–product–derived drugs and 5% were from NP themselves (Clardy & Walsh, 2004). Many of the natural product based drugs currently on the market originated from soil based sources and by continuing to screen terrestrial based ecosystems the likelihood is that the same classes of antibiotics will continue to re–discovered, thus it make sense that other environments such as marine ecosystems should be screened as a potential source for new microorganism that produce bioactive compounds with potential biopharmaceutical applications (Baker *et al.*, 2007).

1.4.1 Isolation strategies for novel bacteria

It is now well established that over 70% of the earth's surface is covered with water and that life evolved in the seas billions of years ago. The marine environment is as yet largely underexplored from a biotechnological perspective, it is widely believed that the diversity of

the marine environment is such that it is likely to be a rich source of microbial biodiversity (Thakur *et al.*, 2008); (Imhoff *et al.*, 2011). However molecular analyses of marine environments suggest that many marine microbes remain uncharacterised and are not prone to cultivation.

If the natural microbial biodiversity is to be assessed and ultimately exploited then traditional culturing techniques will need to be augmented with culturing conditions that mimic more closely the natural environment from which the bacterium was originally isolated. There have been some recent advances in the regard with new experimental new culture conditions such as GigaMatrix (Lafferty & Dycaico, 2004) and Microdish (Ingham *et al.*, 2007), being employed which has led to the identification of novel species and genera. Some of the newly described genera which have been isolated from the marine environment include *Salinospora*, *Demequina* and *Lamerjespora* (Bull & Stach, 2007). Hence innovative techniques such as the aforementioned high–throughput cultivation (HTC) approaches combined with the use of components of the natural environment in the isolation media has resulted in some recent progress in the cultivation of marine bacteria (Connon & Giovannoni, 2002). For example a research group in Turkey has recently (Hames-Kocabas & Uzel, 2012), separated bacteria in samples and incubated them individually (Taylor *et al.*, 2009); (Ishøy *et al.*, 2006) thus avoiding repression of the slow–growing strains. They also provided them with minimal media thus allowing the recovery of bacteria that need low nutrient concentrations and long–term incubation to be isolated. Studies have also shown that bacteria grown at low temperatures allow for the recovery of new isolates (Song *et al.*, 2009) while other groups have used microbial entrapment methodologies resulting in an increased recovery of actinomycetes (Kaeberlein *et al.*, 2002).

1.4.2 Molecular strategies for determining microbial diversity

Molecular techniques based on DNA or RNA analyses are typically employed in accessing the microbial biodiversity in marine samples (Hentschel *et al.*, 2001). Molecular tools such as Denaturating Gradient Gel Electrophoresis (DGGE) which is used to separate the amplified 16S rRNA products has been widely used as a tool to detect the phylogenetic "fingerprint" of diverse marine organisms (Li *et al.*, 2006). The biochemical and the ecological role of the microbes that are unknown can be characterised by tracing back the functional genes to genes that are similar in well–known species using fluorescence *in situ* hybridization (FISH) (Friedrich *et al.*, 2001). Numerous variations in the sequence–based techniques and high–throughput strategies (Werner *et al.*, 2012) for accessing the diversity of marine ecosystems have evolved in recent years. Thus it has been possible to gain a greater understanding of the dynamics and overall structure of marine microbial communities, using small subunit ribosomal RNA (SSU rRNA) in conjunction with high–throughput sequencing technologies such as pyrosequencing (Fierer & Lennon, 2011); (Zengler, 2009); (Lozupone & Knight, 2008); (Huse *et al.*, 2008). Scientists from Ireland used pyrosequencing technology and obtained long average read lengths of ~430 bp (V1–V3 region of 16S rRNA gene) that allowed robust resolution

of sequences to genus level (Jackson *et al.*, 2012).

In addition, innovative combinations of employing microarrays together with other molecular based tools have become increasingly useful for predicting the structure–function relationships of complex microbial communities (Wagner *et al.*, 2007). Studies have also identified non–fermenting bacteria using matrix–assisted laser desorption ionization–time–of–flight mass spectrometry (MALDI–TOF MS) for species identification allowing comparisons with 16S rRNA analysis (Mellmann *et al.*, 2008). In addition as previously mentioned genomic (Zhao, 2011) and metagenomic based approaches (Kennedy *et al.*, 2010) have successfully been employed to identify potential gene clusters encoding novel enzymes involved in the biosynthesis of secondary metabolites which will further facilitate the field of marine based drug–discovery (Dionisi *et al.*, 2012).

1.4.3 Marine natural products

In a previous section of this introduction NP were discussed, but the focus here will specifically be on marine based NP. While about 60% of NP which have been discovered to date can be traced back to the marine environment the success rate from a successful clinical application standpoint has been low due to the somewhat limited access to various marine areas and also security of supply (Lam, 2006). The problem of access has however been largely overcome through the use of remotely operated underwater vehicles (ROVs) (Leal *et al.*, 2012). While many earlier studies were based on samples from coastal areas, the use of ROVs has meant that samples can now be obtained from deep sea studies which increase the possibility of uncovering unique microbial diversity, thereby increasing the likelihood of discovering more novel lead drug molecules (Schauer *et al.*, 2010); (Xu *et al.*, 2010).

Given the fact that actinobacteria are known to be a rich source, it is perhaps not surprising therefore that marine actinomycetes have been the focus of many research groups involved in marine biodiscovery (Gulder & Moore, 2009); (Olano *et al.*, 2009). Relatively recently, representatives from 13 new genera of Actinobacteria of marine origin have been isolated, which could prove to be a source of many novel compounds (Fenical & Jensen, 2006); (Sibanda *et al.*, 2010). Some of the structural features of marine NP are unique and hence it is not surprising that their modes of action are unique as well (Zotchev, 2012). Some of the bioactivities which are displayed by marine based NP include anti–bacterial, anticancer, anti–malarial and anti–viral activities amongst others (Prudhomme *et al.*, 2008).

Currently, there are 13 marine NP which are in various phases of the clinical development (Glaser & Mayer, 2009). Table 1.6 provides a list of NP (marine sources) that are currently undergoing pre–clinical and clinical trials.

Table 1.6 : Examples of Marine natural products undergoing clinical trials.

This table is adapted from (Mayer *et al.*, 2010).

Name of the compound	Source	Activity	Company/ Institution
Cytarabine, Ara–C	Marine sponge	Anti–cancer/ Approved	Bedford, Enzon
Vidarabine, Ara–A	Marine sponge	Anti–cancer/Approved	King Pharmaceuticals
Eribulin Mesylate (E7389)	Marine sponge	Anti–cancer/Phase III	Eisai Inc.
Hemiasterlin (E7974)	Marine sponge	Anti–cancer/Phase I	Eisai Inc.
Hemiasterlin	Marine sponge	Anti–cancer/Phase I	Eisai Inc.

1.5 Marine sponges

Marine sponges are one of the ancient multicellular cellular metazoans which have been residing in the oceans since the Precambian era. They are quite simple organisms and occupy 80% of known water bodies. Sponges pump up to 25 m^3 of water one kg sponge per day through their aquiferous system. They have an epidermal layer called the pinacoderm which is composed of pinacocytes (Figure 1.10). Seawater is drawn through porocytes into an area located between the outer and inner cell layers– the so called mesophyl layer of the sponge; which is composed of a network of canal–like structures. The interior body of the sponge is lined by choanocytes which are flagellated cells that create the sponge's water current and use microvilli to filter out particles from the water. Bacteria within the seawater when they are transferred into the mesohyl tissue are either ingested by archaeocytes or survive and become established as part of the sponge–specific microbiota which become enclosed within the mesohyl matrix. In this way marine sponges play host to significant microbial populations which include bacteria, single–celled eukaryotes (microalgae and fungi) and archaea, which play crucial roles in the biology of the host.

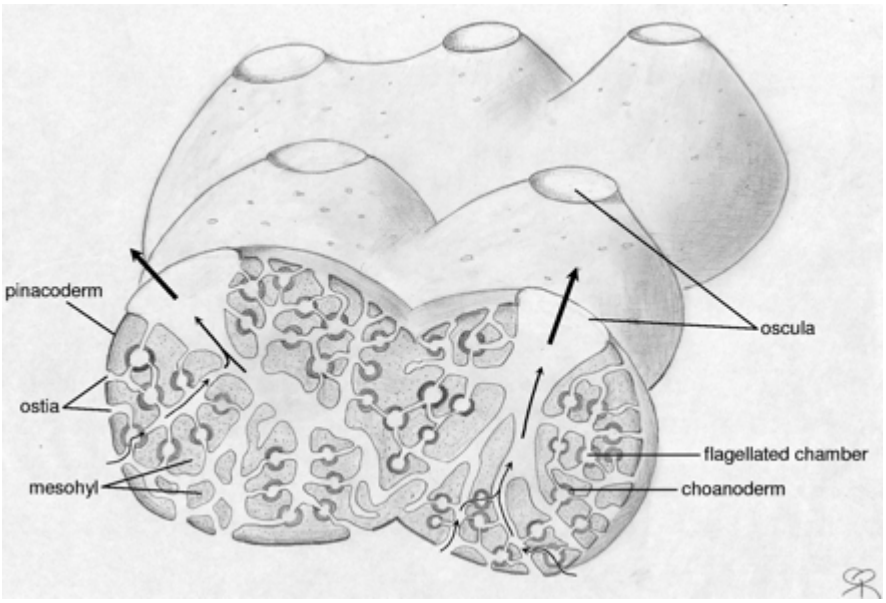

Figure 1.10 : Movement of water flow from a typical marine sponge.

Bacteria along with water enters through the porocytes in the pinocoderm and exists through the osculum.

Adapted from (Taylor *et al.*, 2007).

The sponge microbiota can be symbiotic, pathogenic, act as a food source or be transiently associated with the animal. In some instances, it has been estimated that up to 40–60% of the total biomass of the sponges may comprise endosymbiotic microorganisms, reaching densities as high as between 10^8 and 10^{10} bacteria per gram of sponge wet weight. These are known as high–microbial–abundance (HMA) sponges or bacteriosponges, with a classic example being the Caribbean great barrel sponge *Xestospongia muta* which is believed to contain around 8×10^9 bacteria per gram of sponge tissue. This exceeds the number of bacteria which are typically found in seawater by at least two to four orders of magnitude. Marine sponges are a very good source of NP which many believe may have evolved as a chemical defense mechanism against potential predators and/or to prevent biofouling. More novel bioactive metabolites are obtained annually from sponges than from any other marine taxon. Over 7,000 bioactive compounds have to date been isolated from sponges, many of which have proven to be biologically active and pharmacologically valuable, with anti–cancer, anti–infective and other bioactivities. Many of these NP are structurally very similar and in some cases identical to bacterially derived compounds and it is now widely believed that many of these products are in fact produced by bacterial symbionts of the sponges. Thus understandably there has been a lot of interest in studying the microbiota associated with marine sponges not only from the perspective of potential biotechnological exploitation but also from a microbial ecology perspective, thereby gaining insights into the diverse microbial communities present within the sponge ecosystem.

1.5.1 Microbial diversity associated with sponges

Recent molecular studies has shown that there is a diverse assemblage of bacteria and archae within sponges (Na *et al.*, 2008), while recent pyrosequencing based analysis of sponge microbial communities have revealed diverse sponge–specific microbial communities (Lee *et al.*, 2012); (Jackson *et al.*, 2012). The microbial diversity of sea sponges has recently been described as containing the following 35 bacterial phyla: Acidobacter, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Nitrospira, Proteobacteria (α, β, γ, δ and ε), Deinococcus–Thermus, Firmicutes, Gemmatimonadetes, Planctomycetes, Spirochaetes, Poribacteria, Verrucomicrobia, TM6, Lentisphaerae, BRC1, Chlamydiae, Fibrobacteres, Fusobacteria, Tenericutes,-WS3, Aquificae, Deferribacters, Dictyoglomi, TM7, Chloribi, Chrysiogenetes, OD1, Thermodesulfobacteria, OP10, OS–K, Thermotogae, Synergistetes and Elusimicrobia (Fieseler *et al.*, 2004); (Taylor *et al.*, 2007); (Hardoim *et al.*, 2009); (Webster *et al.*, 2010); (Lee *et al.*, 2011); (Schmitt *et al.*, 2012); (Trindade-Silva *et al.*, 2012).

Several eukaryotes are also known to occur in sponges such as dinoflagellates, parasitic diatoms (Bavestrello *et al.*, 2000), microalgae (primarily *zoochlorellae*) and some fungi (*Aspergillus* sp. and *Penicillium* sp.), with the fungi in particular representing novel biotechnological potential.

1.5.2 Metabolites from sponges and their microbes

The major breakthrough which sparked much of the recent research interest in sponges from a biopharmaceutical perspective was the discovery of the nucleosides spongothymidine and spongouridine from the marine sponge *Cryptotethia crypta*. These subsequently formed the basis for Ara–C, the anticancer drug and the anti–viral drug, Ara–A (Sipkema *et al.*, 2005). Recent studies has also reported brominated indole alkaloids, sesquiterpene quinones and hydroxyquinones isolated from two sponges–*V. rigida* and *S. aurea*. These compounds are potential leads for anti–depressant drugs (Kochanowska *et al.*, 2008). The structures of many sponge–derived NP, and in particular many of the complex polyketides and modified peptides, structurally resemble bacterial compounds. Thus it is a widely held belief that many of these products are in fact produced by bacterial symbionts of the sponges. Examples include cyclodepsipeptide chondramide D isolated from the myxobacterium *Chondromyces crocatus* and jaspamide from the sponge *Jaspis* spp. as well as the myxobacterial metabolite apicularen A which is almost identical to salicylihamide A from *Haliclona* sp. (Erickson *et al.*, 1997).

Many of these sponge–derived bacterial metabolites appear to exhibit several interesting bioactivities such as anti–yeast, anti–fouling, antiangiogenic, antimicrobial and cytotoxic effects. One such example is a metabolite from a novel *Pseudomonas* sp. associated with the sponge *Suberites domuncula*, which is known to produce an antiangiogenic, antimicrobial, and cytotoxic compound (Thakur *et al.*, 2005). Other recent studies have identified a large number of microbes which are associated with sponges which display anti–bacterial and anti–fungal

activities against clinical pathogens (O'Halloran *et al.*, 2011); (Phelan *et al.*, 2012); (Margassery *et al.*, 2012); (Flemer *et al.*, 2012).

1.6 Prospects of Natural Products for drug discovery

If NP are to increase their potential as a possible source of new bioactive molecules with biopharmaceutical potential then a number of obstacles remain to be overcome. Firstly, it will be important to create a more diverse range of compound libraries in order to identify new metabolites from new environments. In addition the genomic and metagenomic experimental datasets which are currently available should be dissected and various bioinformatics based tools (Glöckner & Joint, 2010) should be employed to discover not only potential compounds but also potential new metabolic pathways (Cong *et al.*, 2012). In this respect microbes associated with marine sponges may prove to be a useful starting point for these types of studies, given the track record of success to date in this area. It is also clear that culture based exploitation of marine actinomycetes in particular is likely to lead to the discovery of many potential novel bioactives. New approaches such as the recent mass spectrometry–guided genome mining method that can identify NP and their biosynthetic gene clusters will greatly aid in this respect (Kersten *et al.*, 2011). Cho and co–workers have suggested the use of a combined target and genome–based approach that would help to identify small molecules and their target protein (Cho & Kwon, 2012). While the rediscovery of known compounds (dereplication) will also continue to be a potential problem, modern NMR and MS based methodologies involving data integration software using predicted spectra for many known NP are being developed, which will greatly aid in this process. In addition, early stage dereplication informed by microbial systematics is also now proving to be successful (Vynne *et al.*, 2012).

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Chapter 2

Diversity and antibacterial activity of bacteria isolated from the coastal marine sponges *Amphilectus fucorum* and *Eurypon major* [1]

2.1 Abstract

The aim of this study is to access the bacterial diversity and antimicrobial activity of culturable bacteria associated with two temperate–water marine sponges, *Amphilectus fucorum* and *Eurypon major*. It was found that Proteobacteria were the dominant group of bacteria cultured from both sponges, but overall, the bacterial composition was diverse and distinct between the sponges. The most notable features were the higher proportion of firmicutes in *E. major* and the low frequency of Actinobacteria in both sponges. Four bacterial isolates were identified as potentially novel species and will be characterised in future studies. Approximately 400 cultured bacteria were screened for antimicrobial activity against a collection of indicator strains, with only eight strains, all *Pseudovibrio* spp. displaying any such activity. These strains were active against *Escherichia coli* and *Bacillus subtilis* but not *Staphylococcus aureus* or a selection of fungal strains.

2.2 Introduction

Sponges are primitive metazoans in the order Porifera and are a part of the benthic community that plays a vital role in marine ecology. They are common and widely dispersed in most marine waters and have been considered a source of bioactive metabolites with therapeutic

[1] Published: (Margassery *et al.*, 2012)

potential for some time (Radjasa *et al.*, 2011). Sponges form close associations with a wide variety of bacteria (Webster & Taylor, 2012), which can in some cases constitute up to 40% of the sponge biomass (Grozdanov & Hentschel, 2007). These sponge–associated bacterial symbionts have been postulated to perform a number of roles in the biology of the sponge including the elimination of toxic metabolic by–products, increased structural rigidity, and the production of bioactive metabolites, which may be of use to the host (Taylor *et al.*, 2007). Some of the bioactive compounds that have been isolated from sponges are believed to originate from resident bacteria rather than from the sponge, a fact that has led to a large increase in studies that explore the diversity and ecology of sponge–associated bacteria (Taylor *et al.*, 2007). The microbial diversity in sponges has recently been reported to comprise of at least 35 phyla and a number of studies have been performed in which sponge–associated bacteria were cultured and screened for the production of bioactive compounds (Anand *et al.*, 2006); (Grozdanov & Hentschel, 2007); (Cragg *et al.*, 2009); (Kennedy *et al.*, 2009); (Abdelmohsen *et al.*, 2010); (O'Halloran *et al.*, 2011). The aim of this study was to culture and identify bacteria associated with the Irish coastal sponges, *Amphilectus fucorum* and *Eurypon major*, and to assess the diversity and bioactive potential of these bacteria.

2.3 Materials and Methods

2.3.1 Sponge collection and processing

The sponges *Amphilectus fucorum* and *Eurypon major* were collected by scuba diving from Lough Hyne, a sea lough on the south–west coast of County Cork, Ireland (51°30'N 9°18´W), at depths of 8 m and 15 m respectively, in August 2008 (Kennedy *et al.*, 2009). Detailed descriptions of this Lough and its sponge ecology have previously been published (Bell & Barnes, 2003).

2.3.2 Isolation of bacteria

To remove the contaminants from sea water, the sponge sample was washed in sterile artificial sea water (ASW). On site, 1 g of sponge sample was chopped finely and vortexed with 3 mm glass beads. The sponge homogenate was then serially diluted using ASW and 100 µl aliquots were plated out on isolation media supplemented with 30 µg amphotericin B/ml to inhibit fungal growth. Three different media were used: SYP–SW medium (10 g starch/l, 4 g yeast extract/l, 2 g peptone/l, 33.3 g Instant Ocean/l (Atkinson & Bingman, 1998), 15 g agar/l); Chitin medium (4 g colloidal chitin/l, 33.3 g Instant Ocean/l, 15 g agar/ l; MMA medium (50 µg yeast extract/l, 500 µg tryptone/l, 100 µg sodium glycerol phosphate/l, 33.3 g Instant Ocean/l, 15 g/l). These plates were incubated at 18°C for 4–6 weeks, with colonies subsequently selected for culturing based on morphological features such as colony colour and appearance. Pure cultures were then sub–cultured on SYP–SW plates and stocks were stored at -80°C for long

term usage. All chemicals were purchased from Sigma with the exception of Instant Ocean (Fish Antics, Dublin, Ireland).

2.3.3 Antimicrobial assays

A deferred antagonism assay was used to determine whether bacteria isolated from sponges possessed antimicrobial activity (Kennedy *et al.*, 2009). In brief, bacteria to be tested were grown in SYP–SW agar media at 28°C to a colony size of 0.5–1cm in diameter and then overlaid with soft agar seeded with bacterial or fungal indicator strains. The bacterial test strains were grown overnight and 50 µl of the culture was added to the LB soft agar. All the bacterial strains was diluted to a final OD_{600}=0.1. For the fungal test strains, similar procedure was followed but 50 µl of the culture was added on to YEPD soft agar. Incubation was continued at 28°C and a zone of clearance around the bacteria indicates that it is producing an antimicrobial compound to which the test strain is sensitive. The fungal test strains, which were pre–grown in standard YEPD (10 g yeast extract/l, 20 g peptone/l and 20 g dextrose/l) broth at 30°C, were *Candida albicans* SC5314, *Candida glabrata* CBS138, *Saccharomyces cerevisiae* BY4741, *Kluyveromyces marxianus* CBS6556 and *Aspergillus fumigatus* Af293). The bacterial test strains, which were pre–grown in standard LB broth at 37°C, were *Bacillus subtilis* IE32, *E. coli* 12210 and *Staphylococcus aureus* NC000949.

2.3.4 DNA extraction and PCR Analysis

Bacterial genomic DNA was isolated from fresh colonies or frozen glycerol stocks as follows: either 25 µl of glycerol stock cultures or a single colony was picked and added to 100 µl TE buffer, which was then heated at 98°C for 30 min. The mixture was then centrifuged at 3913×g in an eppendorf microfuge for 15 min at 4°C and 3 µl of the supernatant was used to provide template DNA for PCR. PCR was performed with a total volume of 30 µl containing sterile water, 1X Taq buffer, 2 mM dNTPs, template DNA, Taq DNA polymerase (0.75 U) (Fermentas), and the universal primers (10 µM each) 27F (5´ AGAGTTTGATCCTGGCTCAG 3´), 1492R (5' GGTTACCTTGTTACGACTT 3') (Turner *et al.*, 1999). These primers amplify the 16S rRNA gene, generating a 1.5 kb PCR product. The conditions were as follows: initial denaturation (95°C for 5 min), followed by 36 cycles of denaturation (94°C for 30 sec), primer annealing (50°C for 30sec) and primer extension (72°C for 2 min), completed with a final primer extension step (72°C for 10 min).

2.3.5 DNA Sequencing and Phylogenetic analysis

The amplified PCR products were concentrated using an evaporator (GeneVac–MiVac Duo Concentrator) and sequencing was performed by Macrogen Inc. Korea. Sequences were trimmed (to between 400 and 600 nt depending on the dataset) using FinchTV (`http://`

www.geospiza.com/Products/finchtv.shtml) and then dereplicated using the fastgroup database with a cut–off value of 98.5% (Seguritan & Rohwer, 2001). Sequence similarity searches were performed using BLAST with reference sequences being downloaded from the Ribosomal Database Project (Wang *et al.*, 2007). Sequences were then aligned using MEGA4 software and a phylogenetic tree was constructed using this software (Tamura *et al.*, 2007). The evolutionary history was inferred using the Neighbour–Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The 16S rRNA gene sequences for the isolates were deposited in GenBank under the accession numbers JN039179–JN039214 for *Amphilectus fucorum* and JN040567–JN040606, JN616284 for *Eurypon major*.

2.4 Results

2.4.1 Microbial diversity of the sponges

To try to assess a broad spectrum of bacterial diversity in the two sponges *Amphilectus fucorum* and *Eurypon major*, tissue extracts from these sponges were inoculated onto three different media, namely SYP–SW, Chitin medium and MMA. 335 strains were selected based on morphological differences and microbial diversity was subsequently analysed using 16S rRNA gene sequencing to identify species and genera. Using Fastgroup algorithm (methods), these strains were grouped into 76 taxa (based on a 98.5% 16S rRNA cut off) and one representative from each group was selected for further analysis. The overall culturable microbial diversity of the two sponges is shown in pie chart format (Figure 2.1).

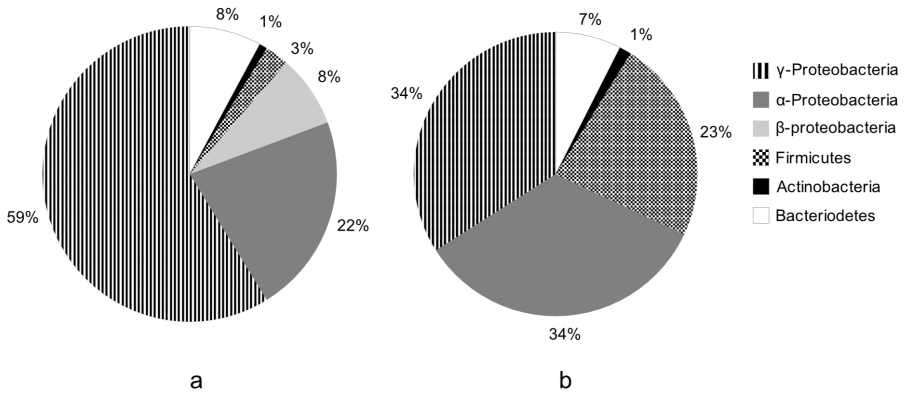

Figure 2.1 : Distribution of the bacterial phyla cultured from a) *Amphilectus fucorum* and b) *Eurypon major*.
16S rRNA sequencing of 335 bacterial strains cultured from the two sponges identified 257 unique 16S rRNA sequences (110 *A. fucorum* isolates and 147 *E. major* isolates). These were grouped using the Fastgroup programme used to classify bacteria by phylum. The relative distribution (as a percentage) of phyla in each sponge is illustrated.

For both sponges, Proteobacteria were the dominant group by far: 89% of the *Amphilectus* isolates (Figure 2.1a) and 68% of the *Eurypon* isolates (Figure 2.1b). The Proteobacterial diversity was studied in more detail by aligning sequences with references and construction of a phylogenetic tree (Figure 2.2). The bulk of strains cultured were α and γ Proteobacteria and although isolates with identical 16S rRNA sequences were not recovered from sponges, there were not striking differences in the patterns or groups of Proteobacteria isolated from both sponges. The most notable feature was the recovery of just one β–Proteobacteria taxon from *Amphilectus*, with no β–Proteobacteria recovered from *Eurypon*. This β–Proteobacteria taxon was recovered 8 times and the phylogeny indicates that it is likely to represent a species within the genus *Ralstonia* (Figure 2.2).

Figure 2.2 : Phylogenetic tree representing Proteobacteria phyla cultured from both sponges.

The evolutionary relationships of the Proteobacterial taxa identified among Proteobacteria cultured in both sponges are shown with reference sequences from RDP and Genbank included. The analysis is based on the 16S rDNA sequence and aligns a single representative sequence for each group identified by the Fastgroup program (methods). The number of similar sequences that is represented by each sequence is shown in brackets, as is the media in which the species was isolated (methods). Cultured isolates from *Amphilectus fucorum* and *Eurypon major* are represented by ▲and ♦ respectively. The neighbour–joining tree was drawn using the MEGA program and bootstrapping percentages (1000 replicates) above 50% are shown. Sequences that may represent novel species are highlighted in bold.

When comparing other groups, it was seen that Bacteriodetes were isolated at similar frequencies in both sponges (7–8%) whereas the proportion of Firmicutes was considerably higher in *Eurypon* (23%) than *Amphilectus* (3%). Actinobacteria were rarely isolated from either sponge (~1%). As with the Proteobacteria, detailed phylogenetic analysis does not identify any specific patterns within other bacterial phyla, although the much higher representation of Firmicutes in *Eurypon* is apparent (Figure 2.3).

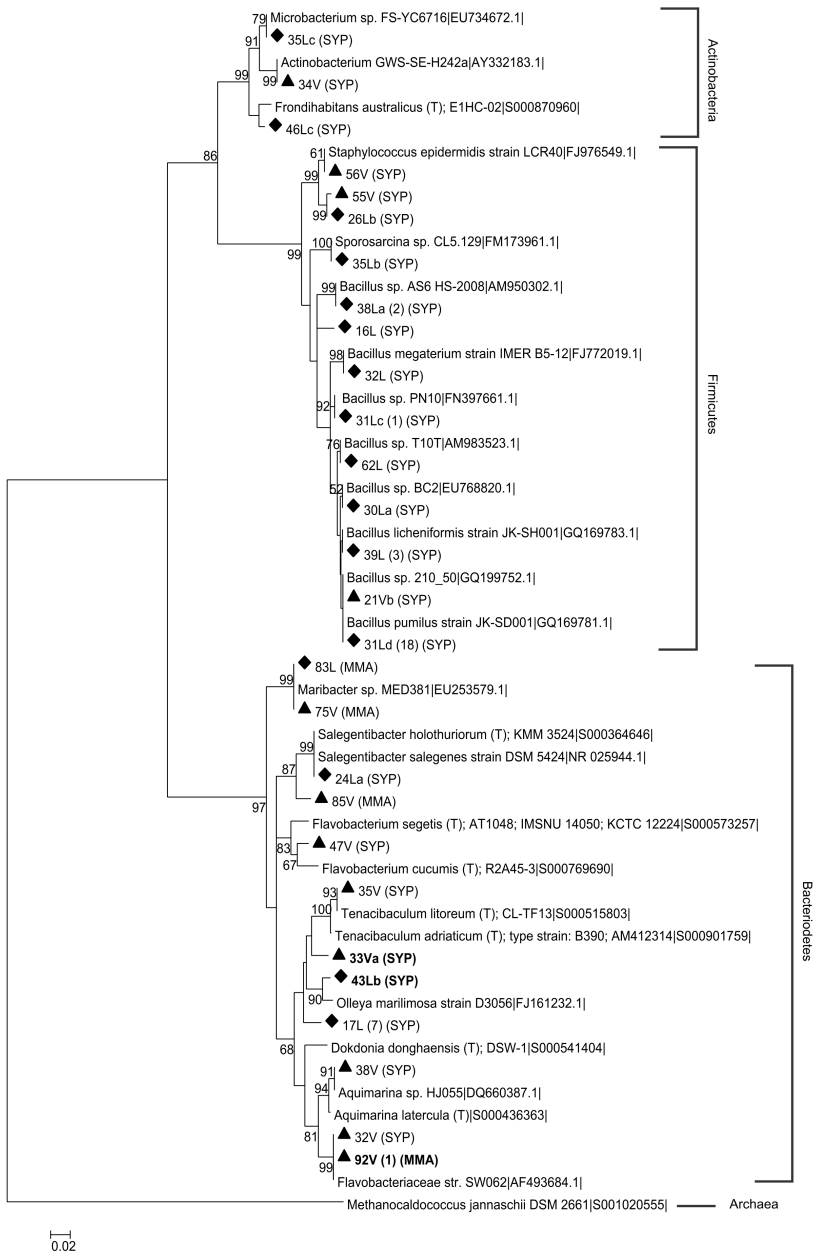

Figure 2.3 : Phylogenetic tree representing the non–Proteobacteria phyla cultured from both sponges

The evolutionary relationships of the the Firmicutes, Actinobacteria and Bacteriodetes taxa identified in both sponges are shown with reference sequences from RDP and Genbank included. The analysis is based on the 16S rDNA sequence and aligns a single representative sequence for each group identified by the Fastgroup program (methods). The number of similar sequences that is represented by each sequence is shown in brackets, as is the media in which the species was isolated (methods). Cultured isolates from *Amphilectus fucorum* and *Eurypon major* are represented by ▲ and ♦ respectively. The neighbour–joining tree was drawn using the MEGA program and bootstrapping percentages (1000 replicates) above 50% are shown. Sequences that may represent novel species are highlighted in bold.

Multiple different *Bacilli* sequence–types are present, suggesting that these spore–forming bacteria may be common in *Eurypon*. Most of the bacterial isolates displayed 100% identity to their related species in BLAST searches indicating that they are isolates of established species. There were four exceptions to this, with two of the *Amphilectus fucorum* isolates and two of the *Eurypon major* isolates (represented in bold in Figure 2.2 and Figure 2.3) showed 97% or less identity to their nearest neighbours. These may represent novel species and further studies are being carried out to classify and characterise these bacteria.

2.4.2 Antimicrobial activity of sponge–associated bacteria

A total of 409 bacterial isolates were tested for anti–fungal activity against *C. albicans*, *C. glabrata*, *S. cerevisiae*, *K. marxianus* and *A. fumigatus* and for anti–bacterial activity against *E. coli*, *B. subtilis* and *S. aureus* using a deferred antagonism assay. None of the isolates displayed any anti–fungal activity but five of the isolates from *Amphilectus fucorum* and three isolates from *Eurypon major* displayed activity (Table 2.1). Isolates 113V, 107L, 108L and 109L inhibited both *E. coli* and *B. subtilus* and a further four strains inhibited one or the other of these test bacteria. None of the isolates displayed anti–staphylococcal activity under these assay conditions. Interestingly, all the active isolates were *Pseudovibrio* sp. and in fact, 8 of the 12 *Pseudovibrio* strains isolated in this study displayed anti–bacterial activity, indicating that such activity is common in the genus.

Table 2.1 : Isolates showing antimicrobial activity against test bacterial strains[1]

Name of the sponge	Sponge isolates	Test strains			
		***E. coli* 12210**	***B. subtilis* IE32**	***S. aureus* NC000949**	**Fungal strains[2]**
Amphilectus fucorum	117V	+	–	–	–
	113V	+	+	–	–
	115V	+	–	–	–
	112V	+	–	–	–
	83V1	–	+	–	–
Eurypon major	107L	+	+	–	–
	108L	+	+	–	–
	109L	+	+	–	–

[1]Activity detected using a deferred antagonism assay.

[2]Five fungal strains tested as described in methods.

2.5 Discussion

In this study, it has been seen that in overall terms, the diversity of culturable bacteria, and dominance of Proteobacteria is similar to that which has been reported for other Irish marine sponges such as *Haliclona simulans* (Kennedy *et al.*, 2009). Actinobacteria were rarely isolated from either sponge (~1%), a finding that contrasts with studies with other sponges such as *Haliclona simulans* (Kennedy *et al.*, 2009). This low frequency of Actinobacteria could indicate that this group is less frequently found in *Amphilectus fucorum* and *Eurypon major* than *Haliclona simulans* but it should be noted that the methodology for isolating bacteria was not the same in both studies: in particular, this study did not use any medium that was specifically selective for Actinobacteria, such as SYP with Nalidixic acid (Kennedy *et al.*, 2009). It must be considered that the medium used will influence the cultured diversity; for example, in this study, whereas α and γ Proteobacteria were isolated on all 3 media used, Firmicutes were only recovered on SYP–SW medium (Figure 2.4, Table 2.2).

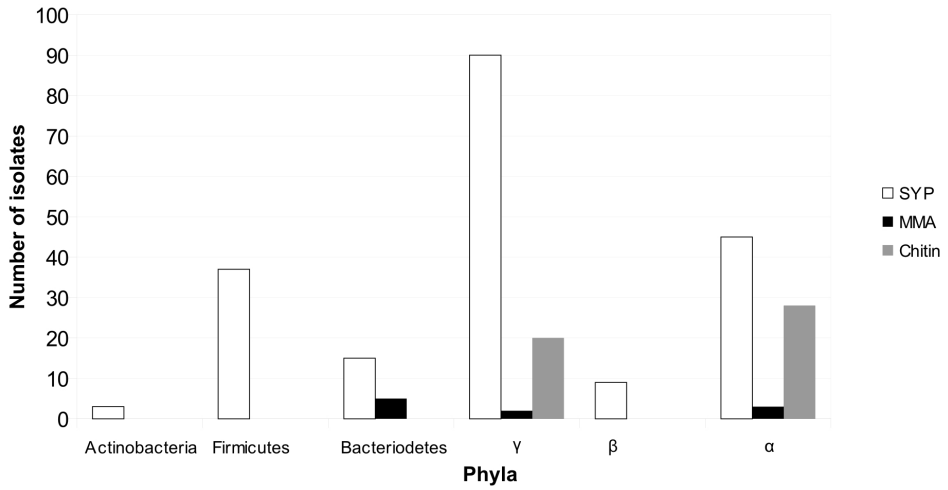

Figure 2.4 : Phyla isolation from different media.

The chart shows the phyla distribution from each of the three media SYP, MMA and Chitin. Interestingly, although Proteobacteria were isolated on all three media, no Bacteriodetes were isolated on chitin agar and Firmicutes and Actinobacteria were isolated only from SYP.

Table 2.2 : Number of isolates recovered from each phyla.

Phyla	Media	Number of isolates
Actinobacteria	SYP	3
Firmicutes	SYP	37
Bacteriodetes	SYP	15
	MMA	9
γ Proteobacteria	SYP	90
	MMA	2
	Chitin	20
β Proteobacteria	SYP	9
α Proteobacteria	SYP	45
	MMA	3
	Chitin	28

It was observed that 8 of the 12 *Pseudovibrio* isolates from the sponges showed anti–bacterial activity which is in accordance with the previous studies which also reported anti–bacterial activity from *Pseudovibrio* sp. against *E. coli*, *B. subtilis*, *B. cereus* and *S. aureus* (Sertan-de Guzman *et al.*, 2007); (Kennedy *et al.*, 2009); (Santos *et al.*, 2010) as well as *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Enterococcus faecium* and *Enterococcus faecalis* (Santos *et al.*, 2010). Furthermore, *Pseudovibrio* sp. isolated from three Irish marine sponges, *Polymastia boletiformis*, *Axinella dissimilis* and *Haliclona simulans*, have also previously been reported to display activity against a number of important pathogens including *Clostridium perfringens* and *Yersenia enterocolitica* (O'Halloran

et al., 2011). Studies have reported that the roseobacter from the order Rhodobacterales produces a novel tropolone antibiotic, tropodithietic acid (TDA) that maintain symbiosis with their algal hosts. Recently, scientists from the US have identified that the expression of this antibiotic is controlled by a novel autoinducer and the genes that are important for the production of TDA was also identified in *Pseudovibro* sp. suggesting that the marine *Pseudovibrio* strains isolated from this study could also be producing TDA (Geng & Belas, 2010). It is interesting that all the *Pseudovibrio* were recovered in chitin agar (Figure 2.4). Furthermore, production of secondary metabolites or other antimicrobials is often tightly regulated, and thus it may be that, for some bacteria, the conditions in this particular assay did not induce their synthesis.

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Chapter 3

Aquimarina amphilectae sp. nov., isolated from the Irish sponge *Amphilectus fucorum* [1]

3.1 Abstract

A gram negative, rod–shaped orange–coloured, catalase and oxidase positive, non–motile bacterium designated as 92V^T was isolated from the marine sponge, *Amphilectus fucorum*, collected from Lough Hyne, Co. Cork, Ireland. 16S rRNA gene sequence analysis revealed that strain 92V^{T}clustered with members of the family Flavobacteriaceae, the closest member being *Aquimarina latercula*, with a gene sequence similarity of 97%. The dominant fatty acids were iso–$C_{17:0}$ 3–OH (38.4%), iso–$C_{17:1}$ ω9c (11.8%) and iso–$C_{15:0}$ 3–OH (10.1%). Combined phenotypic differences and phylogenetic analysis indicates that 92V^T represents a novel species of the genus *Aquimarina*, for which the name *Aquimarina amphilectae* sp. nov is proposed with 92V^T as the type strain (=NCIMB 14726, DSM25232). The GenBank/EMBL/DDBJ accession number for the partial 16S rRNA gene sequence of strain 92V^T is JN039190.

3.2 Introduction

The genus *Aquimarina*, first described in 2005, is classified as yellow pigmented, gram–negative, aerobic, heterotrophic bacteria in the family Flavobacteriaceae (Nedashkovskaya *et al.*, 2005); (Bernardet & Nakagawa, 2006). There has been nine species of *Aquimarina* so far identified from different studies (Nedashkovskaya *et al.*, 2005); (Nedashkovskaya *et al.*, 2006); (Yoon *et al.*, 2006); (Miyazaki *et al.*, 2010); (Yoon *et al.*, 2011); (Yi & Chun, 2011); (Lin *et al.*, 2011); (Park *et al.*, 2011). In this study we report a new strain of *Aquimarina*–like bacterium isolated

[1] Under review

from a marine sponge *Amphilectus fucorum* and the name *Aquimarina amphilectae* sp. nov., is proposed for this novel strain.

3.3 Materials and Methods

3.3.1 Isolation of bacteria

In the study of screening for bioactive producing bacteria, many culturable bacteria were isolated from the marine sponge *Amphilectus fucorum* collected from the Lough Hyne, Co. Cork, Ireland (51°30'N 9°18´W). Strain 92V^{T} was initially isolated from sponge extracts inoculated onto MMA medium (0.005% yeast extract, 0.05% tryptone, 0.01% sodium glycerol phosphate, 3.33% artificial sea salts 'Instant Ocean' brand, 1.5% agar) and incubated at 18°C for 4–6 weeks (Margassery *et al.*, 2012). The strain was subsequently sub–cultured on SYP–SW agar (1% starch, 0.4% yeast extract, 0.2% peptone, 3.33% artificial sea salts 'Instant Ocean' brand) and incubated at 28°C.

3.3.2 DNA extraction and PCR amplification

For PCR amplification of the 16S rRNA gene, bacterial genomic DNA was extracted from fresh colonies to provide template DNA for PCR. PCR amplification was performed with a total volume of 30 µl using Taq DNA polymerase (Fermentas) the 16S rRNA universal primers (10 µM each) 27F (5´ AGAGTTTGATCCTGGCTCAG 3´), 1492R (5' GGTTACCTTGTTACGACTT 3') (Turner *et al.*, 1999) to amplify the near complete 16S rRNA gene. PCR reaction conditions were as follows: initial denaturation (95°C for 5min), followed by 36 cycles of denaturation (94°C for 30 sec), primer annealing (50°C for 30 sec) and primer extension (72°C for 2 min), completed with a final primer extension step (72°C for 10 min). The amplified PCR product was gel purified and sequenced (GATC Biotech). Sequence similarity searches were performed using BLAST with reference sequences being downloaded from the Ribosomal Database Project (Wang, 2006); (Wang *et al.*, 2007). Sequences were then aligned using MEGA4 software and a phylogenetic tree was constructed using this software (Tamura *et al.*, 2007). The evolutionary history was inferred using the Neighbour–Joining method (Saitou & Nei, 1987) and confirmed using maximum likelihood analysis. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

3.3.3 Phenotypic, Biochemical and Chemotaxoniomal tests

The growth of the strain was monitored both under anaerobic and aerobic conditions. Temperature growth range was determined on 2216 plates (Difco) incubated at temperatures ranging from 4–45°C that were monitored for 6 weeks. pH growth range was monitored in marine cytophaga broth containing 4% artificial sea salts (Sigma) with pH ranging from 4–11 and incubated at 25°C for 6 weeks. Penicillin G susceptibility was tested by spreading the isolate onto 2216 plates with a 1 µg penicillin G being placed on the surface. The plates were incubated at 25°C for 7 days and checked periodically for a zone of inhibition.

API enzyme tests were set up with the following modifications. The colonies were suspended in either 0.85% NaCl and an incubation temperature of 25°C was used. As the API strips did not show any indication of growth after 24 h, the incubation temperature was prolonged to 48–72 h. The API ZYM was also increased from 4 h to 20 h. Fatty acid profiles were performed by growing the culture on 2216 media and were analysed using the Sherlock Microbial Identification System by MIDI Inc. The DNA G+C content of the strain 92V^{T} was determined by HPLC (Mesbah *et al.*, 1989); (Tamaoka & Komagata, 1984); (Visuvanathan *et al.*, 1989). All the tests were carried out at The National Collection of Industrial, food and Marine Bacteria (NCIMB).

3.4 Results

3.4.1 Phylogenetic analysis

According to the partial 16S rRNA gene sequence similarity, 92V^{T} was 97% similar to *Aquimarina latercula* strain (Figure 3.1). It was evident that this strain belongs to the *Aquimarina* genus and 16S rRNA gene analysis indicated it was a potential new species. The Genbank accession number for this strain is JN039190.

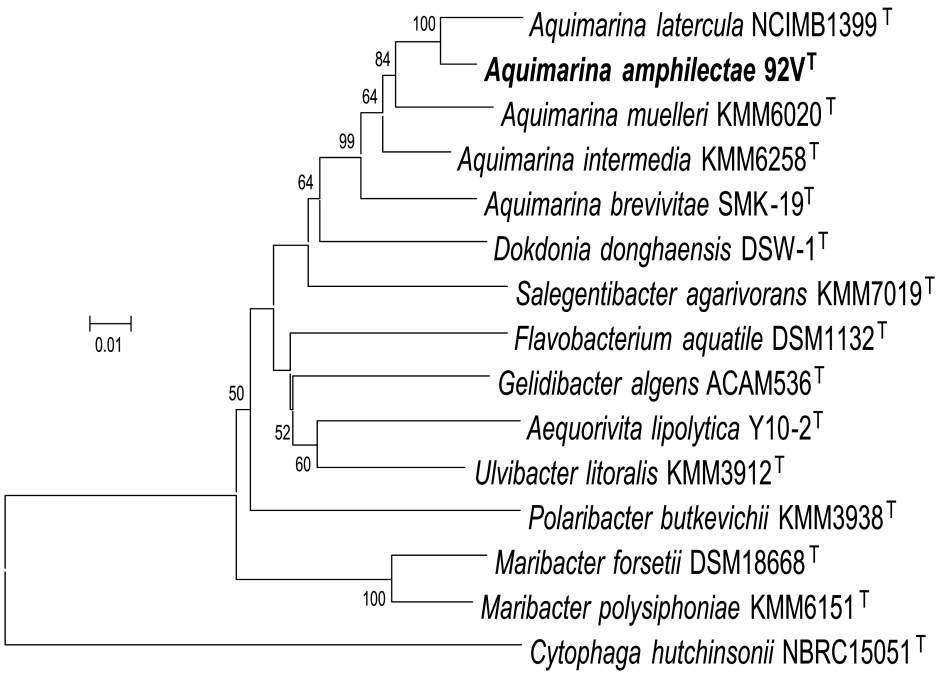

Figure 3.1 : Phylogenetic tree based on 16S rRNA gene sequences of strain and related genera in the family *Flavobacteriaceae*

The tree was constructed using the neighbour–joining method. Numbers at nodes indicate bootstrap values (%). All branches were also maintained in the maximum likelihood tree.

3.4.2 Phenotypic characterisation

The determination of flexirubin type pigments was carried out as described (Bernardet & Nakagawa, 2006); (Bernardet *et al.*, 2002). Other tests such as gram–staining, catalase, oxidase, motility starch and agar hydrolysis, acid production from sugars, hydrolysis of tween80 were investigated as described in previous studies (Healing, 1993); (Johnson & Chilton, 1966). The cellular morphology was examined using the Leica Light Microscope (Leica DM3000) and the cells range from 0.2 µm to 0.25 µm in width and from 2.2 µm to 2.7 µm in length (Figure 3.2).

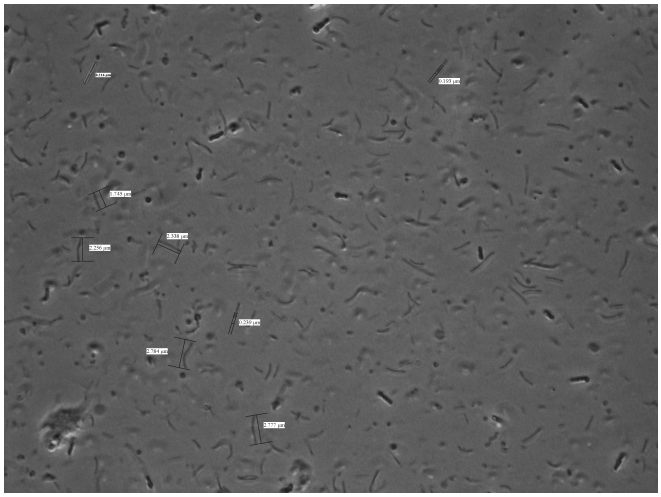

Figure 3.2 : Microscopic examination of the strain 92V^{T}.

The width and the length of the cells are measured and labelled using the in–built Leica LMD software.

The phenotypic, biochemical and chemotaxonomic tests are represented in Table 3.1 and Table 3.2.

Table 3.1 : Phenotypic differences of strain 92V^T with the type strains of other *Aquimarina* species.

Strains: 1, 92V^T; 2, *A. mytili* KCTC 23302; 3, *A. brevivitae* KCTC 12390; 4, *A. intermedia* JCM 13506; 5, *A. latercula* JCM 8515; 6, *A. macrocephali* JCM 15542; 7, *A. muelleri* KCTC 12285; 8, *A. spongiae* KCTC 22663; 9, *A. addita* KACC 14156; 10, *A. agarilytica* NBRC 10695. +, Positive; −, Negative; ND, Not determined. 1 and 5 are from this study. The rest of the data was taken from previous studies (Yi & Chun, 2011); (Lin *et al.*, 2011); (Park *et al.*, 2011).

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Temperature (°C)	10–30	15–37	10–41	4–36	4–34	8–30	4–34	10–35	4–30	4–30
pH	6–10	7–9	5.5–10	ND	ND	ND	ND	6–11	6–8	6–9
NaCl range (%)	2–5	1–4	1–10	1–10	1–5	1–4	1–8	1–5	3–7	1–4
Anaerobic growth	−	−	−	−	−	−	−	−	−	−
Catalase activity	+	+	+	+	−	+	+	+	+	+
Oxidase activity	+	+	+	+	+	+	+	+	+	+
Alkaline phosphatase activity	+	+	ND	+	+	ND	+	+	+	+
Gliding motility	−	+	+	+	−	+	+	−	−	−
Flexirubin–type pigments	−	−	−	+	−	−	−	−	−	−
Nitrate reduction	−	+	+	−	+	+	−	+	+	−
H_2S production	−	−	−	+	+	−	−	−	−	−
Acid production from glucose	−	−	−	−	−	+	−	−	−	+
Penicillin G resistance	+	+	+	−	+	+	+	−	ND	−
Hydrolysis of:										
Agar	−	−	−	−	+	−	−	−	−	+
Aesculin	+	+	+	+	+	−	−	+	+	ND
Tween 80	−	+	+	+	+	−	+	+	+	−
Starch	+	+	+	+	−	+	+	+	+	+
Enzyme activities:										
N–Acetyl–β–glucosaminidase	+	+	−	−	−	−	−	−	−	ND
α–chymotrypsin	+	−	+	−	+	−	+	+	−	ND
Esterase lipase	+	−	+	−	+	−	+	−	−	ND
β–galactosidase	+	−	−	+	+	−	−	−	−	+
β–glucosidase	+	−	+	−	−	−	−	−	−	ND
Trypsin	+	−	+	−	−	−	−	−	+	ND
Valine arylamidase	+	+	+	+	+	−	+	+	+	ND
DNA G+C content (mol %)	36.1	37.9	36	37.1	34	33.1	31.9	36.9	35	32.8

Table 3.2 : Fatty acid compositions (%) of strain 92V^T compared to the other *Aquimarina* strains.

Strains: 1, 92V^T; 2, *A. mytili* KCTC 23302; 3, *A.brevivitae* KCTC 12390; 4, *A.intermedia* JCM 13506; 5, *A.latercula* JCM 8515; 6, *A.macrocephali* JCM 15542; 7, *A.muelleri* KCTC 12285; 8, *A.spongiae* KCTC 22663; 9, *A.addita* KACC 14156; 10, *A.agarilytica* NBRC 10695. +, Positive; –, Negative; ND, Not determined; Tr– Trace. 1 and 5 are from this study. The rest of the data was taken from previous studies (Yi & Chun, 2011); (Lin *et al.*, 2011); (Park *et al.*, 2011).

Fatty acid	1	2	3	4	5	6	7	8	9	10
$C_{16:0}$	0.8	3.3	1.0	4.4	1.59	3.9	10.2	1.9	–	5.1
$C_{18:0}$	–	1.7	Tr	3.3	–	–	6.7	1.0	1.8	ND
$C_{17:1}$ ω6c	3.4	–	–	Tr	1.77	–	–	–	1.5	ND
iso–$C_{13:0}$	0.4	Tr	Tr	Tr	–	4.4	1.9	Tr	–	Tr
iso–$C_{15:0}$	12.2	34	21.5	27.1	18.64	16.8	21.1	38.1	13.3	21.6
iso–$C_{16:0}$	3.5	1.4	2.5	1.6	6.85	Tr	–	1.3	1.1	ND
iso–$C_{15:1}$ G	2.6	12.6	12.3	12.5	7.49	8.7	6.1	15.5	5.4	4.3
iso–$C_{16:1}$ H	1.3	–	1.6	1.2	–	–	–	–	–	ND
iso–$C_{17:1}\omega$9c	11.8	7.6	5.9	9.2	7.8	6.0	9.4	8.2	–	ND
$C_{16:0}$ 3–OH	1.7	Tr	1.5	Tr	2.85	14.5	1.5	0.7	1.2	5.4
iso–$C_{15:0}$ 3–OH	10.1	–	4.9	4.5	5.24	4.8	4.7	5.3	6.1	2.4
iso–$C_{16:0}$	2.6	–	2.4	1.1	2.16	–	–	Tr	1.2	Tr
iso–$C_{17:0}$ 3–OH	38.4	22.3	30.4	23.3	27.44	24.4	28.8	18.1	30	15.4

Phylogenetic analysis indicated that 92V^T was a member of the genus *Aquimarina*. The level of 16S rRNA sequence similarity (94–97%) to the other members of this genus indicated that it could be a novel species. Phenotypic analysis testing clearly indicated 92V^T had all the typical characteristics of the genus *Aquimarina* but a unique phenotypic profile compared to other members of this genus. The differences in enzyme reactions as well as the higher percentage of the fatty acid iso–$C_{17:0}$ 3–OH and the various other fatty acids mentioned in Table 3.2 clearly differentiated the new isolate from recognized species of the genus *Aquimarina*.

3.4.3 Description of *Aquimarina amphilectae* sp. nov.

The strain is strictly aerobic, gram–negative, non–sporulating, non–motile rods with parallel ends. Colonies, on 2216 agar plates were shiny, smooth, translucent and raised, and produced a non–diffusible orange pigment (flexirubin negative). The strain grows at 10–30°C (optimum, 25°C) and at pH 6–10 (optimum, pH 6–7). The strain requires sea salt for its growth and it grows on media with artificial sea salts but not on media supplemented with only NaCl.

The strain is catalase, oxidase and alkaline phosphatase positive, is able to hydrolyse starch and aesculin and is resistant to penicillin G. There is no nitrite or nitrate reduction, nor hy-

drolysis of casein, agar or Tween80. Results for indole production, gelatinase, β–galactosidase were variable. The major fatty acid present is iso 3–OH $C_{17:0}$ (38.4 %). Other acids greater than 10% are iso–$C_{15:0}$ (12.2%), iso 3–OH $C_{15:0}$ (10.1%) and iso–$C_{17:1}$ ω9c (11.8%). The DNA G+C content of the type strain $92V^T$ is 36.1 mol%. The type strain, $92V^T$ (= NCIMB 14726), was isolated from the marine sponge, *Amphilectus fucorum*.

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Chapter 4

A high–throughput screen to identify novel calcineurin inhibitors [1]

4.1 Abstract

Calcineurin is a eukaryotic protein phosphatase important for many signalling and developmental processes in cells. Inhibitors of this enzyme are used clinically and there is interest in identifying novel inhibitors for therapeutic applications. This report describes a high–throughput assay that can be used to screen natural or chemical libraries of compounds to identify new calcineurin inhibitors. The microtitre plate assay is based on a yeast reporter strain and was validated with known inhibitors and tested in a pilot screen of bacterial extracts and a sponge metagenomic library.

4.2 Introduction

Calcineurin is a highly conserved, eukaryotic calmodulin–dependent serine/threonine phosphatase type 3CA (formerly PP2B). In mammalian cells, it participates in diverse cellular processes such as signal transduction, cell–cycle regulation, stress response and apoptosis (Rusnak & Mertz, 2000) and has recently been implicated in several important neurodegenerative disorders, including schizophrenia (Kvajo *et al.*, 2010); (Tabarés-Seisdedos & Rubenstein, 2009). In the immune system, calcineurin is centrally involved in maturation of T cells and, for this reason, most immunosuppressive therapies involve the use of calcineurin inhibitors (CNI) (Castroagudín *et al.*, 2011). The most widely used CNIs are cyclosporin A and tacrolimus (FK506), both of which are natural microbial metabolites that bind to intracellu-

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lar receptors known as immunophilins, which then block calcineurin function. The mode of action of and history of these CNIs has been extensively studied and very well–documented (Liu *et al.*, 1991); (Pritchard, 2005). Interestingly, the main details of the mechanism of action of both cylcosporin A and FK506 were first determined in the budding yeast, *S. cerevisiae*, something made possible by the strong conservation of calcium signalling pathways and calcineurin across the Eukarya (Fox & Heitman, 2002). Although CNI are extensively used for immunosuppressive therapy, most notably in organ transplantation, there are cardiovascular, renal and other side–effects that can occur with long–term use (Rezzani, 2006); (Fung *et al.*, 1991) and thus there is interest in identifying novel calcineurin inhibitors. Furthermore, such molecules may also have potential as novel anti–fungal agents (Steinbach *et al.*, 2007) and applications even as cell biology reagents to study calcium signalling and homeostasis are possible.

The most prolific reservoir of bioactive molecules has always been the natural world and the majority of molecules used in medicine are derived from microbes or plants. Many antibiotics, as well as important drugs such as CsA, tacrolimus (FK506) and sirolimus (rapamycin), are of microbial origin and in the new current of genome biology there is renewed interest in bioprospecting to identify new microbial metabolites with therapeutic applications. The challenge of developing screens to identify specific inhibitory activities remains, however, and modern requirements stipulate that any methods are specific, rapid, high–throughput and automatable in so far as is possible. Current methods to screen for calcineurin inhibitors are limited and do not satisfy these requirements (Fruman *et al.*, 1992); (Enz *et al.*, 1994); (Sellar *et al.*, 2006). The aim of this study was to develop a new screen for calcineurin inhibitors that would be compatible with robotic handling systems for screening large numbers of microbial extracts, metagenomic libraries or combinatorial chemistry libraries.

The screen was developed using yeast reporter strains. In yeast, as in mammalian cells, various extracellular signals lead to a transient increase in calcium ions in the cytoplasm. The cytoplasmic protein calmodulin binds calcium and then activates calcineurin. The targets of calcineurin differ among organisms, with the transcription factor Crz1p, the main target in yeast. Following dephosphorylation by calcineurin, Crz1p translocates to the nucleus where it activates transcription of target genes by binding to a promoter sequence known as the CDRE element (Kraus & Heitman, 2003); (Cyert, 2003). The conservation of calcineurin in Eukarya facilitated the development of this yeast screen.

4.3 Materials and Methods

4.3.1 Strains and growth conditions

Saccharomyces cerevisiae BY4741 was used as the standard strain for all assays. Typically, yeast strains were grown in YNB medium at 30°C with appropriate auxotrophic selection to

maintain plasmids (Sherman, 1991). Routine lithium acetate transformation (Gietz *et al.*, 1992) was used to introduce the reporter plasmids pMRK212, which carries a *CRZ1::lacZ* fusion (Serrano *et al.*, 2002), and pRSP97, which carries a *GFP::CRZ1* fusion (Polizotto & Cyert, 2001).

4.3.2 Development of the assay

To prepare yeast cells for the assay, the strain carrying the *CRZ1::lacZ* fusion was grown overnight and inoculated into fresh medium at an A_{600} of 0.1. The fresh culture was allowed to undergo 2–3 doublings (approximately 5 h) before cells were harvested by centrifugation and resuspended at an A_{600} of 0.4. 25 µl of this cell suspension followed by 25 µl of the test reagent (or control solution) was added to each well of a 96–well polystyrene micro titre flat bottomed plate and the plate incubated at 30°C for 30 min. Next, alkaline stress is provided by adding 25 µl of TAPS (N–Tris(hydroxymethyl)methyl–3–aminopropanesulfonic acid) pH 8.1 (100 mM stock, final concentration, 33.3 mM) and incubating at 30°C for 1 h. A β–galactosidase activity assay was then performed as follows. 75 µl of Z–buffer of pH 7.0 (60 mM disodium hydrogen phosphate, 40 mM Sodium dihydrogen phosphate, 10 mM KCl, 1 mM Magnesium sulphate heptahydrate) was added. Cell lysis was by addition 75 µl of lysis buffer (1% N–lauroylsarcosine and 0.5% β–mercaptoethanol in Z–buffer) and incubation for 30 min at 30°C. The use of N–Lauroyl sarcosine for cell lysis was to avoid the effect of chloroform on the plastic microtitre plates (Kippert, 1995). Lysis was followed by the addition of 25 µl ONPG solution to each well and incubation at 30°C until a yellow colour developed in the positive control (typically 20 mins). The time was recorded and the reaction stopped by addition of 60 µl of 1 M Na_2CO_3. The A_{405} was then measured in a plate reader (Thermo Multiskan FC) and units calculated. Arbitrary units were defined as follows: Activity = [$A_{405} \times 1000$] / [Time (min) $\times$ Volume of the cells (ml)]. The A_{600} of the cells is not included in the formula as experience showed that although the assay is highly reproducible with respect to the relative values of positive and negative controls, absolute values can vary and all data must be compared to these controls within an experiment. For comparison, all values were then normalised with the negative control (typically ~150 units) given a value of 1.

4.3.3 Testing and validation of the assay

All assays include a well to which no TAPS is added and this serves as the base–line from which an increase in expression following alkaline stress is observed. All other wells have TAPS added as described above and so should see an increase in expression of the *CDRE::lacZ* reporter. One well simply has 25 µl control solution (medium or organic solvent as appropriate to the test) and this is the positive control for the assay, whereas all the test wells have solutions or metabolites pre–added in advance of the TAPS (above). To validate the system, the effects of three known calcineurin inhibitors: FK–506 (Tacrolimus), Cyclosporin A and Ascomycin as well as three other inhibitors/anti–fungal metabolites: Okadaic acid, Ra-

pamycin (Sirolimus) and fluconazole, were tested. Each of these reagents prepared in appropriate stocks and added to a final concentration as follows: FK–506 (1.5 µg/ml) Cyclosporin A (25 µM), Ascomcyin (0.004 µM), Rapamycin (0.78 µM) and Okadaic acid (1.56 µM), Fluconazole (0.125 mM). With the exception of fluconazole (Sigma), all other test reagents were purchased from LC Laboratories, USA. Following validation, a pilot screen was performed with 81 bacterial extracts. The bacteria used were largely marine–sponge associated bacteria cultured as part of a larger marine biodiscovery project (Kennedy *et al.*, 2009). The bacteria were cultured for 7–14 days in SYP broth (10 g starch/l, 4 g yeast extract/l, 2 g peptone/l, 33.3 g Instant Ocean/l) to obtain supernatant. These extracts were prepared from bacterial culture supernatant by treating cell–free supernatant with amberlite XAD–16 resin for 2–4 h, which was then washed with water and eluted with methanol. This concentrated methanol extract was diluted 1 in 10 with YNB media for the assay. All experiments are carried out in triplicate.

4.3.4 Screening of calcineurin inhibitors from a sponge metagenomic library

The metagenomic library of the marine sponge, *Haliclona simulans* was constructed as described previously (Lejon *et al.*, 2011). The total community DNA was extracted from the microbiota associated with the sponge *Haliclona simulans* which was collected from from the Kilkieran bay, Galway coast on the west coast of Ireland, at a depth of 15 m (N 53°18.944´, W 09°40.140´) in August 2007. It was size–fractionated using pulse–field–gel–electrophoresis and an insert of ~40kb was cloned into a fosmid Copy Control pCC1FOS™ vector to construct a metagenomic library. The library constituted approximately 48,000 clones. The clones were transferred to 384–well plates containing the storage media (10 g tryptone/l, 5 g yeast extract/l, 5 g NaCl/l, 6.3 g KH_2PO_4/l, 1.8 g KH_2PO_4/l, 0.5 g trisodium citrate/l, 0.9g ammonium sulphate/l, 0.09 g $MgSO_4.7H_2O$/l, 60 ml glyerol/l) using the QPIX2–XT robot after an in vitro packaging of lambda phages and infection of *E. coli* TransforMax™ EPI300™. The clones that were stored in −80°C in a 384–well plate were thawed and replicated onto another 384–well plate using the robot containing LB supplemented with 12.5 µg chloramphenicol/ml. Following overnight incubation 37°C, the clones were replicated on to 96 deep–well plates containing LB supplemented with 12.5 µg chloramphenicol/ml and 0.01% arabinose for increasing the copy number and incubated at 37°C overnight. Approximately 3840 clones were screened by the high–throughput assay described above to determine whether they carried genes encoding proteins that were able to inhibit the calcineurin pathway.

To prepare the extract, clones were lysed *in situ* by treating with 1 mg lysozyme/ml for 15 min followed by three freeze–thaw cycles at an interval of 15 min for each step. The plates were then centrifuged at 6104×g for 15 min and the supernatant was taken as the test extract for the calcineurin assay. The calcineurin assay was performed by adding 25 µl as mentioned above. Simultaneously, a control *E. coli* strain with no insert was treated as above.

4.3.5 Characterisation of metagenomic clones

Eight fosmids (HS02P13, HS03E21, HS03I9, HS04F24, HS07C7, HS07F21, HS07N21, HS88E5) were sequenced using 454 pyrosequencing (Roche, GS FLX$^+$ system) with the pool of other clones. Both the sequencing and assembly were done at the University of Liverpool, Centre for Genomic Research. The contigs for all the fosmids were assembled using the RAMMCAP pipeline (Li, 2009) hosted by CAMERA (Sun *et al.*, 2011). ORF predictions were analysed and refined by blastp hosted by NCBI.

The clone HS02P13 was studied in detail to identify the genes that were responsible for the inhibition of the calcineurin pathway. Conventional gene cloning using restriction enzymes BsiWI, HindIII, PmlI, NheI and AatII were performed. The fosmids were self–ligated and individual inserts were transformed into an *E. coli* strain EPI300 and selected in LB supplemented with 12.5 µg chloramphenicol/ml. The genes of interest (predicted protein, phosphatidyl serine decarboxylase, NADH:ubiquinone oxidoredcutase M and N subunit, NADH:ubiquinone oxidoredcutase N subunit and NADH:ubiquinone oxidoredcutase L subunit) were digested with KasI, EcoRI, BglII and SphI respectively. Sub–cloning of the various genes into pLITMUS 39 (Evans *et al.*, 1995) was performed by linearizing the vector (KasI, EcoRI, BamHI and SphI) and treating with alkaline phosphatase (New England Biolabs) to prevent the recircularization of the vector. Each of the plasmid containing various genes were transformed into XL–10 Gold Ultracompetent *E. coli* cells (Stratagene), and the clones were selected in LB supplemented with 100 µg carbenicillin/ml. The plasmids from 10 individual clones from each of the sub–cloning experiments were isolated using the alkali–lysis method (Fermentas kit). These were analysed using restriction digestion with the restriction enzymes mentioned above to ascertain if the genes were properly inserted. Two clones from each experiment comprising the genes mentioned above were screened for anti–calcineurin activity as described in Section 4.3.2. The gene that was not screened initially was the hypothetical protein (as confirmed by blastp). Hence an additional sub–cloning was done by digesting the gene using MfeI and linearizing the vector using the same enzyme. Cloning was done as mentioned above and two clones were tested for anti–calcineurin activity. All the restriction enzymes were purchased from Fermentas.

4.3.6 Fluorescence Microscopy

Epifluorescent microscopy was used to visualize the *GFP::Crz1p* fusion protein in yeast cells with or without alkaline stress and with or without inhibition of calcineurin. Strain BY4741 carrying the pRSP97 reporter plasmid was grown to mid–log phase (A_{600}= ~0.8) in YNB media at 30°C. Cultures were split and exposed to alkaline stress by treating with 30 mM KOH (or control treatment). Individual cultures were also contemporaneously treated with FK506 or putative calcineurin inhibitors to observe the effect on Crz1p localization. Following addition of KOH and the putative drug incubation followed for 15–30 min at 30°C in a dark room

before cells were visualised by epifluorescent microscopy. For visualization, 25 µl of each sample is taken and visualized with DIC and GFP using an I3 filter (emission of 450–490 nm and excitation of 510 nm). The image is then visualized and captured by using the Leica Florescence Microscope.

4.4 Results

4.4.1 Development of a high–throughput assay of calcineurin activity

The basis of the high–throughput assay to identify calcineurin inhibitors developed in this study is that calcineurin activity can be detected by the activity of a *CDRE::lacZ* gene fusion, which is carried on the pMRK212 plasmid. A cytoplasmic calcium spike is triggered by alkaline stress (addition of TAPS buffer at pH 8.1) and this in turn activates calmodulin, calcineurin and Crz1p, which then binds to the CDRE element on the reporter construct activating expression. Expression of the *CDRE::lacZ* gene fusion can be detected using a modified assay for the β–galactosidase enzyme. A major criterion for the assay was that it could be performed in a 96 well microtitre plate, thereby being amenable to HT robotic methods if desired. Although all individual aspects of the system are routine in yeast, integration into a single assay required various modification and optimizations mentioned in the methods. This culminated in the high–throughput assay illustrated in Figure 4.1.

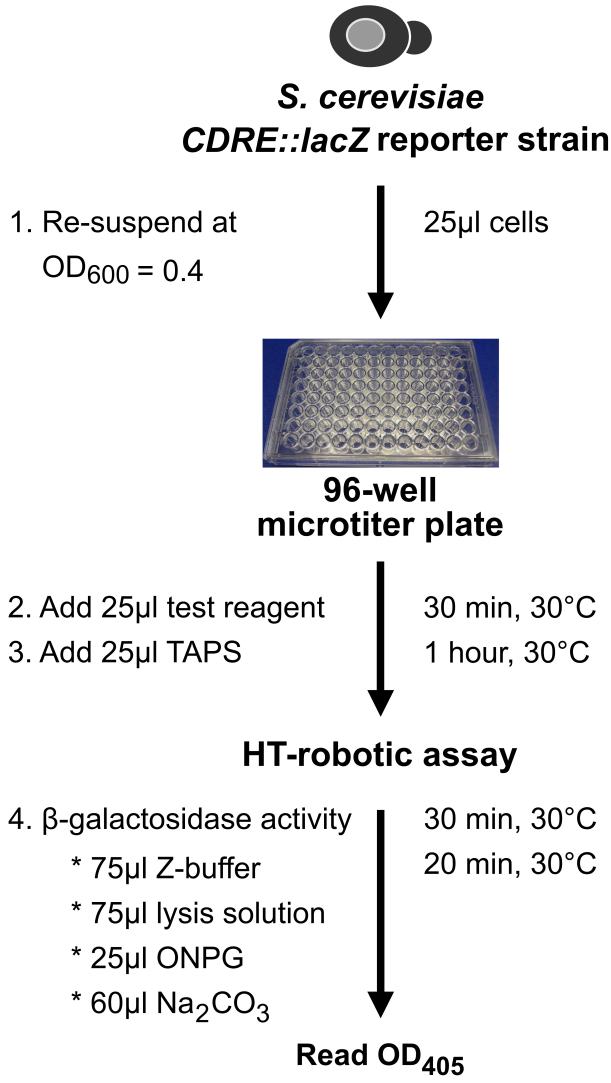

Figure 4.1 : Overview of a scalable assay to identify calcineurin inhibitors.

The diagram provides an overview of the method used to assay for calcineurin inhibitors. The key feature is that calcium–mediated induction of β–galactosidase in the reporter strain requires calcineurin activity. Any molecule that inhibits this activity will prevent the alkali shock (TAPS)–mediated induction of expression and this is detected by an enzymatic assay. All steps of the assay can be performed by a robotic system.

The specificity of the assay was validated by assessing the effects of known calcineurin inhibitors as well as inhibitors of other classes of phosphatase and anti–fungal compounds (Figure 4.2). The induction of *CDRE::lacZ* expression by alkali stimulation is seen by the increase from lane 1 to lane 2. The inclusion of the calcineurin inhibitors FK506 (lane 3), ascomycin (lane 4) or cyclosporin A (lane 5) prevents the induction, thereby demonstrating how calcineurin inhibitors can be identified. It is important for the assay that the effect is specific to calcineurin inhibition, therefore Rapamycin, an immunosuppressive drug with a distinct mode of action from CNIs (lane 6) and Okadaic acid, which inhibits the class of PP2A phos-

phatases (lane 7) and were also tested. Neither of these potent molecules prevented induction of *CDRE::lacZ* expression. In addition, it was confirmed that the assay is independent of inhibitory effects on yeast growth; for example, inhibitory effects of the anti–fungal drug fluconazole has no effect on the assay (data not shown).

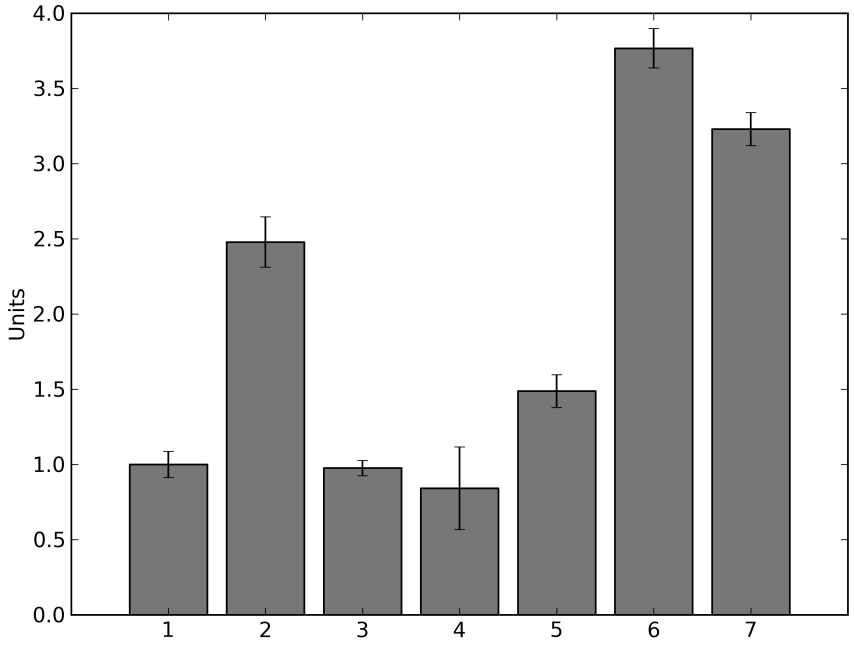

Figure 4.2 : Validation of the calcineurin inhibition assay.

The calcineurin inhibition assay was carried out in a microtite plate as indicated in Figure 4.1. Different treatments, including known calcineurin inhibitors and other classes of inhibitory molecules, were included as indicated in each lane. With the exception of lane 1 (negative control), all treatments included stimulation by addition of TAPS, pH 8.1. Lane 1, negative control, no stimulation; lane 2, positive control; lane 3, FK–506; lane 4, ascomycin; lane 5, cyclosporin A; lane 6, rapamycin; lane 7, okadaic acid. The effect of the known calcineurin inhibitors is seen in lanes 3–5, whereas unrelated inhibitors have no effect on the assay (lanes 6 & 7). Assays were carried out in triplicate.

4.4.2 Application of the calcineurin inhibition assay in a pilot screen

A pilot screen was carried out to validate the assay and to demonstrate its applicability for high–throughput approaches. A library of extracts of marine sponge–associated bacteria was available for this. One microtitre plate that contained methanol extracts from 81 different bacteria was tested to identify candidate calcineurin inhibitors. From this assay, three candidate extracts with potential calcineurin inhibition were identified. This is illustrated in Figure 4.3, where data for examples of two non–inhibitory extracts (lanes 3 and 4) as well as the three inhibitory extracts (lanes 5–7) are presented. The other 76 non–inhibitory extracts followed the pattern seen in lanes 3 and 4 (data not shown).

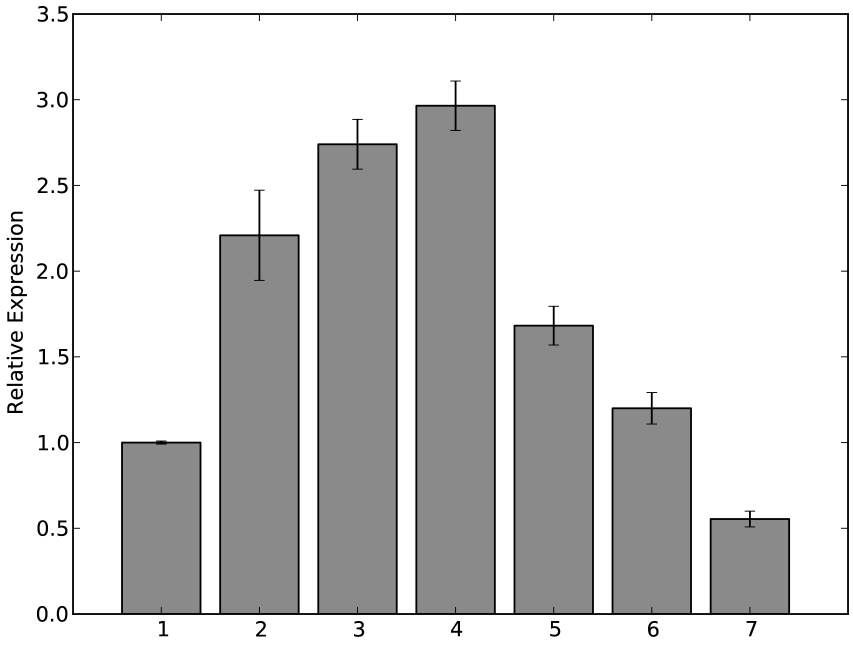

Figure 4.3 : Pilot screen to identify calcineurin inhibitors in a library of bacterial extracts.

A library of 81 bacterial extracts was tested in a pilot screen to identify candidate calcineurin inhibitors. The assay was as described in Figure 4.1, with controls as described in Figure 4.2. With the exception of lane 1 (negative control), all treatments included TAPS alkaline shock and all treatments included the same solvent (10% methanol). 78 extracts that were negative are represented by lanes 3 and 4, and data from the three positive extracts are shown in lanes 5–7. Extracts are denoted by library numbers. Lane 1, negative control, no stimulation; lane 2, positive control; lane 3, 24 N; lane 4, 20; lane 5, SM8; lane 6, FMK7A; lane 7, FMK1B. It is seen that the relative expression in lanes 3 and 4, exceeds that of the positive control (lane 2) whereas relative expression in lanes 5–7 is more similar to that of the negative control (lane 1). Assays were carried out in triplicate.

4.4.3 Metagenomic library screening

A second pilot screen was carried out to determine whether the method was suitable for screening metagenomic libraries (Figure 4.4). A metagenomic library that constituted of 48,000 clones of *Haliclona simulans* was constructed as mentioned previously (Lejon *et al.*, 2011).

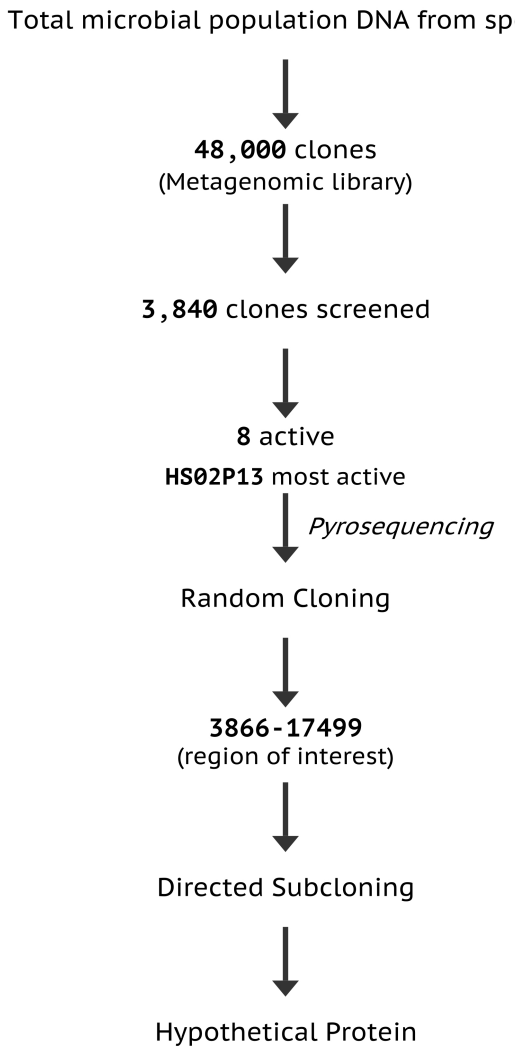

Figure 4.4 : Strategy to identify putative calcineurin inhibitor genes in a sponge metagenomic library.

The metagenomic library of microbiota associated with *H. simulans* was screened to identify potential inhibitors that block the calcineurin pathway. Eight putative clones were identified and sequenced. The clone HS02P13 that showed the best inhibition was subjected to cloning strategies and a hypothetical protein was identified as a putative candidate.

Approximately 3840 clones from this metagenomic library were screened for anti–calcineurin activity using the high–throughput screen developed. Eight clones were found to inhibit the calcineurin pathway in *S. cerevisiae* (Figure 4.5).

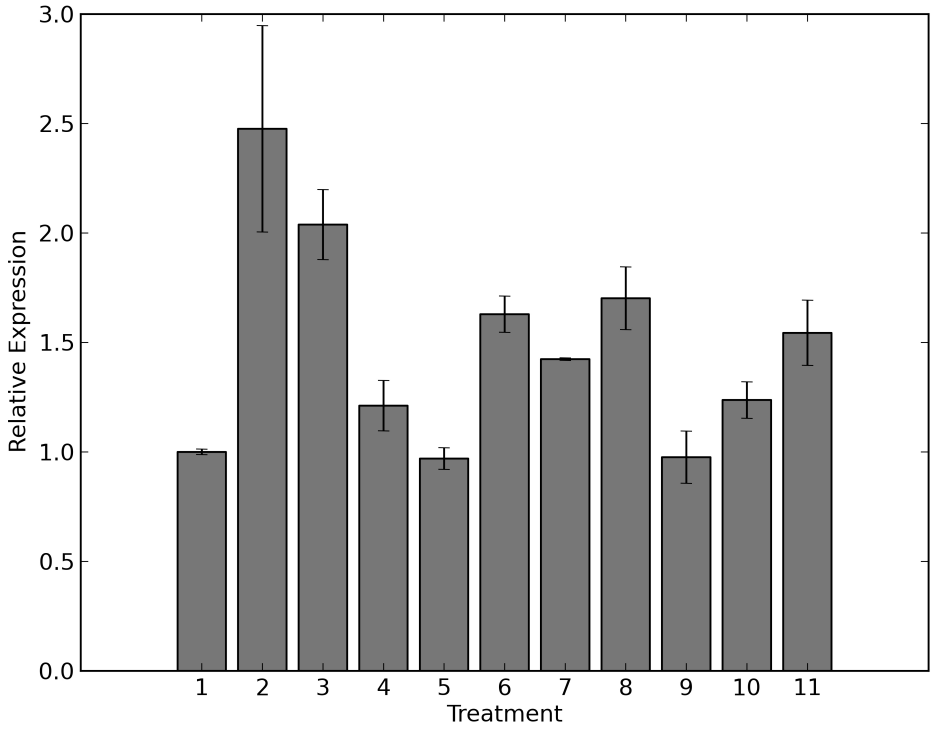

Figure 4.5 : Metagenomic sponge library screening.

A sponge metagenomic library of approximately 3840 clones was screened to identify putative clones that showed calcineurin inhibition. The assay was as described in Figure 4.1, with controls containing the lysate from an *E. coli* strain without any insert represented in lanes 1 and 2. With the exception of lane 1 (negative control), all treatments included TAPS alkaline shock. 3832 clones are represented by lane 3 and data from the eight positive clones are shown in lanes 4–11. The extracts are denoted by library numbers. Lane 1 negative control, no stimulation; lane 2, positive control; lane 3, HS01M5; lane 4–11 (HS02P13, HS03E21, HS03I9, HS04F24, HS07C7, HS07F21, HS07N21, HS88E5) respectively. It is seen that the relative expression in lane 3 is similar to that of the positive control (lane 2) whereas relative expression in lanes 4–11 is more similar to that of the negative control (lane 1). Assays were carried out in triplicate.

These fosmid clones were sequenced using 454 pyrosequencing and were assembled using the RAMMCAP pipeline hosted by CAMERA. The annotation was obtained only from four of the fosmids and among the data obtained, the clone that showed the most anti–calcineurin activity from the assay was HS02P13 (Figure 4.5, lane4). Hence this was selected for analysis. Gene prediction was carried out with GeneMark™ (`http://exon.gatech.edu/GeneMark/metagenome/`), a gene prediction software that uses refined heuristic models (Zhu *et al.*, 2010). This resulted in the identification of 27 coding regions. A BLAST search was done to predict the putative proteins encoded by these genes (Table 4.1).

Table 4.1 : Predicted genes and proteins in clone HS02P13.

Gene ID	Start	Stop	Name of the protein
1	1693	2	Xanthine dehydrogenase family protein (large)
2	2184	1690	Xanthine dehydrogenase family protein (small)
3	3041	2181	Xanthine dehydrogenase family protein (medium)
4	3329	3715	Transposase
5	4074	3712	Putative PLP–dependent enzyme
6	5227	5093	Conserved hypothetical protein
7	5226	6989	Hypothetical protein
8	7633	8616	Phosphatidyl serine decaroboxylase
9	9071	10276	Carbamoyl phosphate synthase, small subunit
10	10726	13356	Carbamoyl phosphate synthase, large subunit
11	15356	13899	NADH–quinone oxidoreductase, N subunit
12	15421	15353	Hypothetical protein
13	16968	15424	NADH–quinone oxidoreductase, M subunit
14	18945	16981	NADH–quinone oxidoreductase, L subunit
15	19316	19011	NADH–quinone oxidoreductase, K subunit
16	19933	19313	NADH–ubiquinone/plastoquinone oxidoreductase chain 6
17	19933	20421	NADH–quinone oxidoreductase, chain I
18	21482	20430	NADH–quinone oxidoreductase, H subunit
19	23953	21497	NADH–quinone oxidoreductase, G subunit
20	25305	23983	NADH–quinone oxidoreductase, F subunit
21	25932	25387	NADH–quinone oxidoreductase, E subunit
22	27185	25932	NADH–quinone oxidoreductase, D subunit
23	27960	27178	NADH–quinone oxidoreductase, C subunit
24	28500	28024	NADH–quinone oxidoreductase, β subunit
25	28847	28491	NADH–quinone oxidoreductase subunit 3
26	29364	28987	NADH–quinone oxidoreductase subunit 1
27	34564	29687	Hypothetical protein, partial

4.4.3.1 Random cloning of HS02P13

Conventional gene cloning using restriction enzymes BsiWI, HindIII, PmlI, NheI and AatII was performed. The fosmids were self–ligated and individual inserts were transformed into an *E. coli* strain. Five clones were then screened for anti–calcineurin activity. The region of interest was between 3866 and 17499 bp (Figure 4.6).

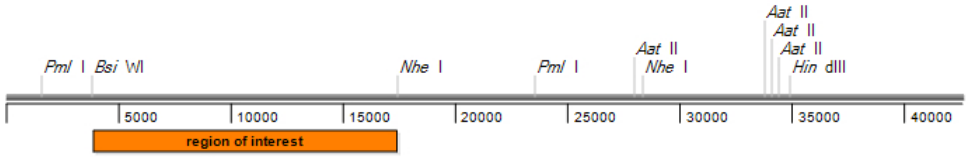

Figure 4.6 : Linear map of the clone HS02P13 highlighting the region of interest.

The clone was analysed both by single (AatII, PmlI and NheI) and double digestions (NheI/PmlI, HindIII/BSiWI) and the region that showed calcineurin inhibition was between 3866 and 17499bp.

4.4.3.2 Sub–cloning strategy

Virtual mapping was carried out for all regions using the Lasergene software (Figure 4.7). The promoter was predicted using the bioinformatics software called Softberry BPROM that can be accessed online (`http://linux1.softberry.com/berry.phtml`). These fragments were cloned into pLITMUS 39 as described in the methods (Section 4.3.5).

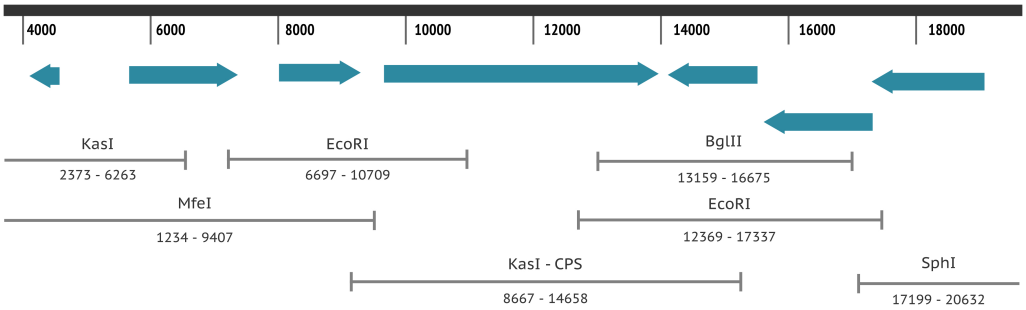

Figure 4.7 : Various genes encoded in the region that showed calcineurin inhibition.

The blue arrow indicates the direction in which the genes are transcribed. Restriction enzymes are designed to clone the fragments individually and screen them for calcineurin inhibition. From left to right, the proteins that are cloned are: conserved hypothetical protein, hypothetical protein, carbamoyl phosphate synthase, NADH–quinone oxidoreductase N subunit, NADH–quinone oxidoreductase M and N subunit and NADH–quinone oxidoreductase L subunit.

The clones were isolated and restriction analysis was performed to check the inserts and it was tested using the calcineurin assay. The only region that showed inhibition in the assay was a hypothetical protein (Figure 4.8).

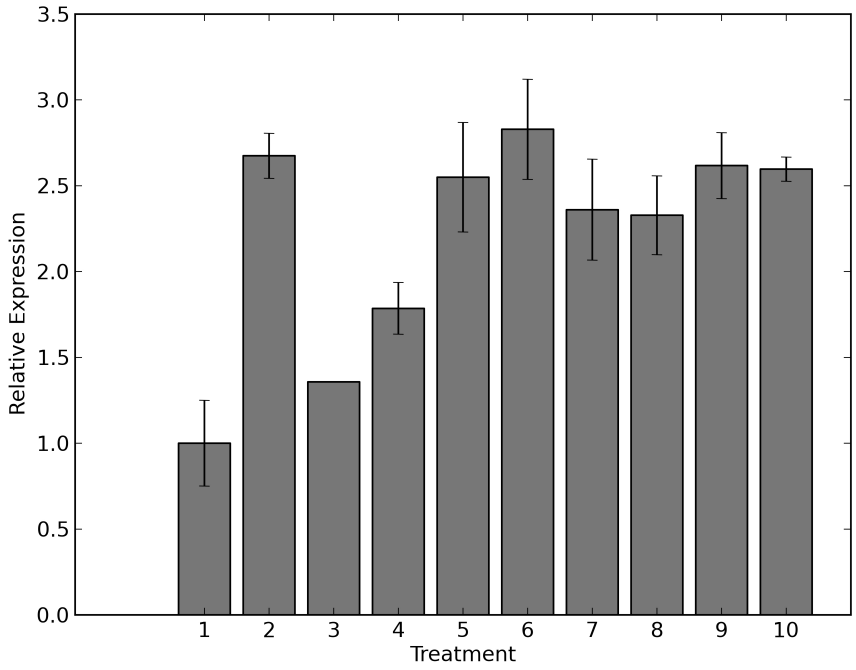

Figure 4.8 : Hypothetical protein showing calcineurin inhibition.

The assay was as described in Figure 4.1, with controls as described in Figure 4.5. With the exception of lane 1 (negative control), all treatments included TAPS alkaline shock. Lane 1 negative control, no stimulation; lane 2, positive control; lane 3, HS02P13 (positive control); lane 4, lysate from hypothetical protein; lane 5, lysate from conserved hypothetical protein; lane 6, lysate from carbamoyl phosphate synthase; lane 7, lysate from phosphatidyl serine decarboxylase; lane 8–10, lysates from NADH::ubiquinone oxidoredcutase (M and N, N and L subunits) respectively. It is seen that the relative expression in lanes 5–10 was similar to that of the positive control (lane 2) whereas relative expression in lanes 3 and 4 are more similar to that of the negative control (lane 1). Assays were carried out in triplicate.

The protein was re–analysed using blastp and it showed 29% identity to a Na^+/Ca^{2+} exchanger protein. The query coverage in the blastp to the Na^+/Ca^{2+} exchanger protein (YP_001635005.1) was 67% with an E–value of 4e–15. An alignment was done using ClustalX (`http://www.clustal.org/clustal2/`), (Figure 4.9), (Larkin *et al.*, 2007).

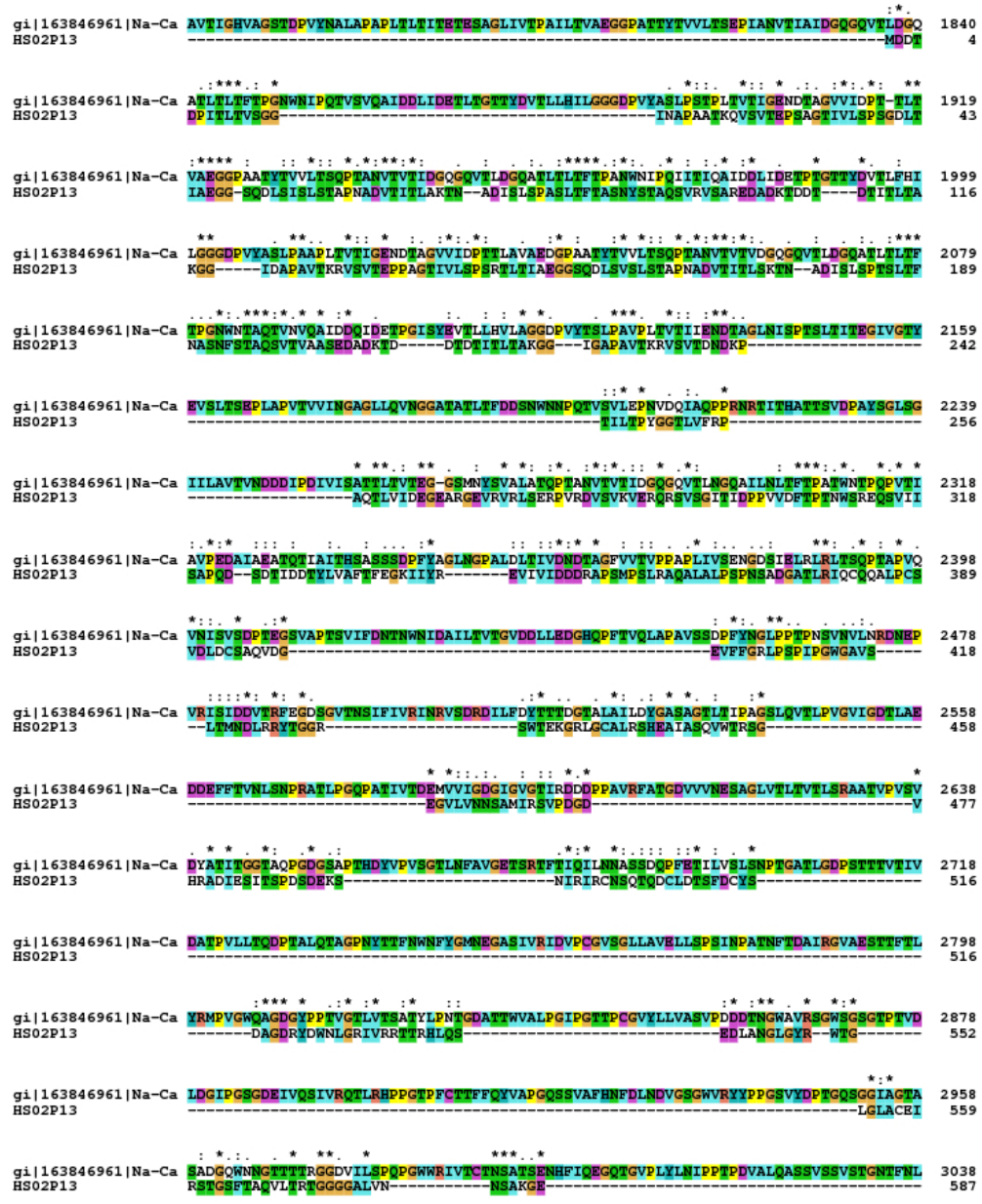

Figure 4.9 : Alignment of HS02P13 with Na^{+}/Ca^{2+} exchanger protein (YP_001635005.1).

The alignment of the hypothetical protein of the clone HS02P13 was done using ClustalX with the Na^{+}/Ca^{2+} exchanger protein.

4.4.4 Localisation assay of Crz1p following calcineurin inhibition

The principle of the screen is that inhibition of calcineurin prevents the translocation of Crz1p to the nucleus that normally follows alkaline stress. To demonstrate that this is in fact the case, and to confirm that the putative inhibitors identified in the pilot screen described above have this effect, another reporter was used. This construct is a *GFP::CRZ1* fusion that has been

demonstrated to accurately report the sub–cellular localization of the Crz1p protein (Polizotto & Cyert, 2001). Crz1p is normally cytoplasmic but following alkaline treatment the protein is dephosphorylated by calcineurin and translocates to the nucleus (Figure 4.10, panels A and B). Addition of either the known calcineurin inhibitor FK–506 (Figure 4.10, panel C) or one of the extracts containing a putative inhibitor (Figure 4.10, panel D) prevents effective translocation and GFP fluoresence throughout the cell was seen. These data demonstrate that the microtitre assay using the *CDRE::lacZ* fusion (Figures 4.2 and 4.3) does successfully identify inhibitors of calcineurin activity. As mentioned above, restricting growth does not have this effect but it is possible that interfering with calcium signalling in another way (e.g. channel blocking or calmodulin impairment) could have similar effects.

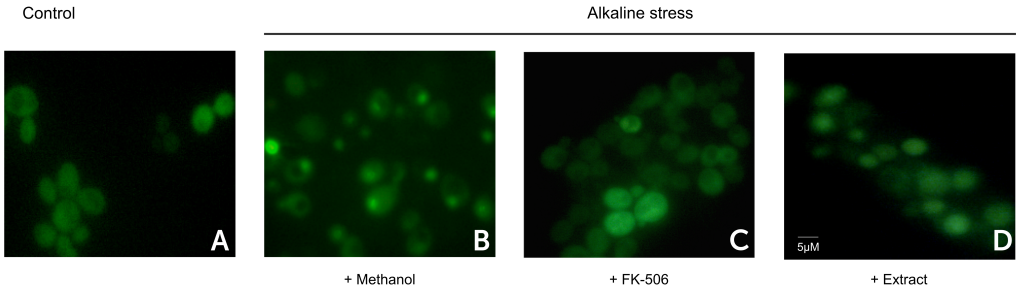

Figure 4.10 : Inhibition of calcineurin prevents Crz1p nuclear localisation.

A yeast reporter strain expressing the *GFP–Crz1p* fusion protein was stimulated by alkaline stress (KOH) and the localisation of the fusion protein monitored by epifluorescent microscopy. Panel A shows cytoplasmic localisation of the protein in unstimulated cells. Panel B shows nuclear localisation following stimulation. As a control, methanol is added in the same concentration and volume as panels C and D. Panel C (FK–506) and panel D (extract from bacterial strain SM8) include metabolites that are known (FK–506) or suspected (SM8) to inhibit calcineurin activity. In both cases, fluorescence is cytoplasmic and the *GFP–Crz1p* fusion fails to localise to the nucleus.

4.5 Discussion

We developed a high–throughput reporter gene based assay that would be compatible with the robotic handling systems for screening. The assay developed is a target–based approach that helps in the identification of new drugs in a shorter duration. This method is simple, reproducible, sensitive and capable of handling large amount of extracts. The limitations of other assays such as the exposure of radioactives in the radioactive–based assay and the time consumption in the colorimetric assay has been overcome by the high–throughput assay.

The metagenomic library screening resulted only in a success rate of `0.2%`. The reason behind such low success rate is because the library is maintained in an *E. coli* host. Studies have revealed that the functional expression of environmental genes in heterologous hosts are poor due to the inefficient transcription of genes therefore an improper assembly of the

corresponding enzymes. Hence for the identification of novel proteins, an efficient expression system is required. The reason behind the inhibition observed by the clone in the metagenomic library is unclear and hence further investigation with the protein should be carried out. As it is an exchanger protein, there is a possibility that this would be sequestering calcium from the cells even before the calcineurin pathway is initiated and hence the result of the inhibition in cells.

Recently, a transposon based method called MuExpress was developed to enhance the expression of protein in *E. coli*. The recombinant transposon randomly inserts into the environmental DNA that facilitates the gene expression from its inducible promoters (Troeschel *et al.*, 2010). Researchers in the USA have constructed tools for functional screening the metagenomic library in *S. lividans* (McMahon *et al.*, 2012). According to their analysis, the bioactive clones were functional only in *S. lividans* but not in *E. coli* which suggests that new surrogate hosts must be constructed and screened for various assay to identify novel bioactive clones.

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Chapter 5

Identification of bioactive metabolites from a *Streptomyces* strain [1]

5.1 Abstract

Marine sponges are a known source of bioactive metabolites but it is predicted that many sponge–derived metabolites are in fact produced by symbiotic microbes. The purpose of this study was to identify bioactives from the bacterial extracts associated with marine sponges obtained from Irish waters. In a screen of 120 bacterial isolates cultured from the sponge *Haliclona simulans*, 25 isolates that inhibited fungi in both deferred–antagonism and disc diffusion assays were identified. Following preliminary analysis, one particular marine specific *Streptomyces* strain, SM8 that produces a low molecular weight metabolite(s) and that can be extracted by organic solvent was selected for further studies. This strain secretes an activity that is inhibitory to yeasts and fungi, including the pathogens *C. albicans* and *A. fumigatus*. These metabolites were purified by solid–phase extraction and reverse–phase HPLC with fractions assayed for antimicrobial activity using the NCCLS assay. NMR analysis led to the identification of butenolides exhibiting anti–calcineurin activity. This would be the first report of a butenolide from any source showing this activity. A number of antimycin–like molecules, uncharacterised anti–bacterial metabolites and a hydroxylated saturated fatty acid were also identified in the extract.

[1] Manuscript in preparation

5.2 Introduction

The marine ecosystem is known to have a diverse microbial diversity and biotechnological potential as 70% of the earth's surface is occupied by water (Gandhimathi *et al.*, 2008). Marine sponges play an important role in the benthic community as they act as a reservoir for a diverse microbial community (Paul *et al.*, 2011). During evolution, these simple, sessile filter feeders developed resistance to protect them from harmful organisms. Studies have revealed that they have physical scaffolds which can help in the development of bacterial communities (Friedrich *et al.*, 2001). The reported symbiotic functions of these microbes include stabilisation of the sponge skeleton, processing metabolic waste, nutrient acquisition and production of secondary metabolites (Taylor *et al.*, 2007); (Thomas *et al.*, 2010). The secondary metabolites produced by the microbes may act as a chemical defence for the sponges. Many metabolites produced by these microbes are known to possess certain biotechnological potential and further exploitation of these metabolites may lead to the discovery of new drugs (Sipkema *et al.*, 2005).

Drug–resistant microbes have become a common cause of death and morbidity in both immunocompetent and immunocompromised individuals (Armstrong-James & Harrison, 2012). The medical field is facing challenges to treat tumours, organ transplantations and immune disorders. It has been seen that effective treatments for microbial infections are often lacking and the financial costs to these healthcare systems is often staggering (Steinbach *et al.*, 2007). The increase in antibiotic resistance among clinical microbes has created a need to develop antimicrobials with novel modes of action. Studies relating to sponge–associated microbes have often led to the discovery of novel metabolites or even known metabolites with novel activities. The focus on *Streptomyces* sp. in several studies has sparked an interest due to its wide array of metabolites produced by them. For example: *Streptomyces* sp. strain JBIR–43 exhibited cytotoxic activity and two other strains (JBIR–34 and 35) showed unique chemical skeleton containing non–proteinogenic amino acids and based on the genome mining it was predicted that these were produced by novel NRPS genes (Khan *et al.*, 2011). Recent studies have also reported a novel ansamycin, from a commensal *Streptomyces* sp. showing severe cytotoxic activities (Lu & Shen 2007); (Yang & Li, 2012). Another study reported deoxyuridines from *Streptomyces microflavus* isolated from the marine sponge *Hymeniacidon perlevis* (Li *et al.*, 2011). Scientists from Germany reported an anti–parasitic activity in *Streptomyces* sp. strains isolated from mediterranean sponges (Pimentel-Elardo *et al.*, 2010).

The sponge species that was selected for this study was *Haliclona simulans*, a member of the order Haplosclerida which has been known to produce a wide range of secondary metabolites. Studies have reported the identification of novel metabolites showing cytotoxic and anti–bacterial activity. One of the studies reported novel cyclic metabolite such as dialkylpyridiniums that showed moderate cytotoxic and anti–bacterial activities against Gram–positive strains (Lee *et al.*, 2012). Another group reported two novel cyclic depsipeptides exhibiting anti–inflammatory properties (Randazzo *et al.*, 2001). Scientists from Korea have recently

identified a new macrocyclic diamide from *Haliclona* sp. from Korean waters that exhibited moderate cytotoxic and anti–bacterial activity against diverse microbial strains (Jang *et al.*, 2009). Another study reported the identification of araguspongin C, an anti–fungal compound from the methanol extract of the sponge, *Haliclona exigua* (Lakshmi *et al.*, 2010).

The secondary metabolites synthesised by *Haliclona* sp. are microbial drug candidates isolated from marine invertebrates with a spectrum of activities such as anti–bacterial, anti–fungal and anti–cancer (Piel *et al.*, 2004). One of the studies identified several bacterial strains exhibiting a broad spectrum of activities against both bacterial and fungal test strains (Kennedy *et al.*, 2009). Another study reported a metabolite, tyrocidine A, isolated from a *Vibrio* sp. associated with the *Haliclona* sp. sponge that showed anti–bacterial activity against methicillin resistant *S. aureus*, *E. coli* and *P. aeruginosa* (Noro *et al.*, 2012).

The aim of this study was to identify novel bioactives from the bacteria associated with the marine sponge, *Haliclona simulans*.

5.3 Materials and Methods

5.3.1 Sponge collection, processing and isolation of bacteria

Isolation of bacteria from the sponge *H. simulans* was previously reported (Kennedy *et al.*, 2009). Briefly, the sponge *Haliclona simulans* was collected from the Kilkieran bay, Galway coast on the west coast of Ireland, at a depth of 15 m (N 53°18.944´, W 09°40.140´) in August 2007. To remove contaminants from sea water, the sponge sample was washed in sterile artificial sea water (ASW). On site, 1g of sponge sample was chopped finely and vortexed with 3mm glass beads. The sponge homogenate was then serially diluted using ASW and 100 µl aliquots were plated out on isolation media supplemented with 30µg amphotericin B/ml to inhibit fungal growth. These plates were later incubated at 28°C for 8 weeks in the laboratory, with colonies subsequently selected for culturing based on morphological features such as colony colour and appearance. Pure cultures were then sub–cultured on SYP–SW plates. For long term preservation, the stocks were prepared by growing the culture in SYP–SW broth at 28°C followed by the addition of 15% glycerol to the culture and stored at −80°C. Spore suspension of sporulating bacteria were also stored at −80°C by adding 20% glycerol for long term storage. All chemicals were purchased from Sigma with the exception of Instant Ocean (Fish Antics, Dublin, Ireland).

5.3.2 Isolation and growth media

Five different isolation media were used: SYP–SW medium (10 g starch/l, 4 g yeast extract/l, 2 g peptone/l, 33.3 g Instant Ocean/l (Atkinson & Bingman, 1998), 15 g agar/l); Modified marine agar medium (50 µg yeast extract/l, 500 µg tryptone/l, 100 µg sodium glycerol phosphate/l,

33.3 g Instant Ocean/l, 15g agar/l); Marine Agar (1 g yeast extract/l, 5 g tryptone/l, 33.3 g Instant Ocean/l, 15 g agar/l); Actinomycete isolation seawater agar (22 g Difco actinomycete isolation agar/l, 5 g glycerol/l, 33.3 g Instant Ocean/l, 15 g agar/l) and NaST21Cx (Magarvey *et al.*, 2004). The plates were made with and without 25 µg Nalidixic acid/ml. The strains were isolated after incubating the plates at 28°C for 8 weeks followed by culturing and preservation of bacterial strains as mentioned in the section above.

For the maximal production of secondary metabolites from one of the *Streptomyces* sp. strain SM8, the growth media had to be optimised. Initially, the seed culture was grown in the 410 media (10 g glucose/l, 10 g glycerol/l, 15 g casamino acids/l, 5 g oatmeal/l, 10 g peptone/l, 5 g yeast extract/l, 1 g calcium carbonate/l, 33.3 g Instant Ocean/l) for 7 days at 28°C. Then 1ml of the seed culture was inoculated to 50 ml of the growth media. Five different growth media were used (Goodfellow & Fiedler, 2010): 19 medium (20 g mannitol/l, 20 g peptone/l); 400 medium (10 g glucose/l, 3 g meat extract/l, 3 g peptone/l, 20 g soluble starch/l, 5 g yeast extract/l, 3 g calcium carbonate/l); MMM medium (10 g glucose/l, 5 g tryptone/l, 20 g soluble starch/l, 5 g yeast extract/l, 1 g calcium carbonate/l); OM (20 g oatmeal/l); SGG (10 g glucose/l, 10 g glycerol/l, 2.5 g corn steep powder/l, 5 g peptone/l, 10 g soluble starch/l, 2 g yeast extract/l, 3 g calcium carbonate/l, 1 g sodium chloride/l). The media was prepared with and without Instant Ocean. It should be noted that the strain was able to grow only in the presence of instant ocean which implies that the strain is marine specific and not a contaminant.

5.3.3 Extraction of the antimicrobial compounds

The SM8 seed culture was grown initially in a 410 media for 7 days at 28°C. 16 ml of the seed culture were inoculated into 800 ml of OM media with Instant Ocean. It was grown for 12 days at 28°C in a shaking incubator at 200 rpm. 16 l of the culture were centrifuged at 12074×g for 15 min in a Beckmann Coulter Avanti J–26 centrifuge. The supernatant was passed through miracloth (Calbiochem) to remove the debris. 1.6 l of amberlite XAD–16 resin were added and incubated overnight to increase the efficiency of the compound binding to the resin. To the supernatant, fresh resin was added and incubated for 4 h to allow the residual compound to bind. The resin was washed with methanol to remove the compound completely that was bound to the resin (the methanol was added to the resin until the resin turns white). The methanol extract was concentrated in a Speed Vac. The extract once concentrated was partially purified by a liquid–liquid partitioning with ethyl acetate and water. Using a separating funnel, both the phases were collected and tested for antimicrobial activity using the NCCLS assay (National Committee for Clinical Laboratory Standards).

5.3.4 Antimicrobial assays

5.3.4.1 Deferred antagonism assay

A deferred antagonism assay was used to determine whether bacteria isolated from sponges possessed antimicrobial activity (Kennedy *et al.*, 2009). In brief, bacteria to be tested were grown in SYP–SW agar media at 28°C to a colony size of 0.5–1cm in diameter and then overlaid with soft agar seeded with bacterial or fungal indicator strains. The fungal test strains were grown overnight and diluted to a final OD_{600}=0.1. 50 µl of the overnight culture were seeded to 10 ml of the YEPD soft agar (10 g yeast extract/l, 20 g peptone/l and 20 g dextrose/l, 7 g agar/l). For the bacterial test strains, a similar procedure was followed but 50 µl of the culture were seeded to 10 ml of the LB soft agar (20 g LB/l, 7 g agar/l). Incubation was continued at 28°C and a zone of clearance around the bacteria indicates that it is producing an antimicrobial compound to which the test strain was sensitive. The fungal test strains, that were pre–grown in standard YEPD (10 g yeast extract/l, 20 g peptone/l and 20 g dextrose/l) broth at 28°C, were *Candida albicans* SC5314, *Candida glabrata* CBS138, *Saccharomyces cerevisiae* BY4741, *Kluyveromyces marxianus* CBS6556 and *Aspergillus fumigatus* Af293. The bacterial test strains, that were pre–grown in standard LB broth at 37°C, were *Bacillus subtilis* IE32, *E. coli* 12210, *Staphylococcus aureus* NC000949 and *P. aeruginosa* PA01. All the chemicals were purchased from Sigma.

5.3.4.2 Well–diffusion assay

The bacterial strains that possessed antimicrobial activity in the overlay assay were grown in SYP–SW broth at 28°C (Kennedy *et al.*, 2009). The culture was centrifuged at 6104×g for 10 min and the supernatant was collected for all 7 days, the only exception being the *Streptomyces* sp. as the supernatant was collected daily between 8 and 14 days. The fungal test strains were streaked on the YEPD media to create a lawn of cells and holes were punched using a cork borer. The supernatant was added to the respective wells. The plates were then incubated at 28°C. A similar procedure was carried out for the bacterial test strains where the test strains were streaked on to the LB media and incubated at 37°C.

5.3.4.3 National Committee for Clinical Laboratory Standards (NCCLS)

NCCLS is a quantitative assay that helps to determine the minimum inhibitory concentration (MIC) of the extract (Liu *et al.*, 2007). The growth media used for fungal strains was 10.4 g RPMI–1640/l in 165 mM MOPS and the pH adjusted to 7.0. The principle of the assay is that the dye, resazurin (blue) gets reduced to resorufin (pink) when the cells grow. The dye remains blue when there is inhibition of growth. The test extracts (cell–free supernatant, bacterial extract dissolved in methanol or the XAD–broth) were diluted with the media (depending on the concentration) and added to the first well of the 96–well plate. The other wells are filled

with 100 µl of the media. The test extracts were serially diluted (1:2) up to 10 columns. The last two columns would be the growth and the sterility controls (Figure 5.1). The fungal test strains were initially grown on YEPD agar plates and the colonies were suspended in 2 ml of distilled water at an OD_{540}=0.12 that corresponds to $1–5\times10^6$ CFU/ml. The resazurin cell suspensions were prepared by mixing 100 µl of cells and 250 µl of resazurin (Stock concentration–20 mg resazurin/ml) in 50 ml of RPMI media. 100 µl of this suspension was added to the 96–well plate except to the sterility control. The dye for the sterility control was prepared with a working concentration of 0.1 mg resazurin/ml in 10 ml of RPMI media. 100 µl of this solution was added to the sterility control wells. The plates were incubated at 37°C. The procedure for the bacterial test strains remains the same except the media used for the anti–bacterial assay would be the isosensitest broth (23.4 g isosensitest broth/l, Purchased from Oxoid). The bacterial test strains were grown in 5 ml LB overnight at 37°C. The overnight bacterial strains were then re–inoculated in to 50 ml of LB and grown until the OD_{600}=1. 100 µl of cells were used for the assay as mentioned above. All chemicals are purchased from Sigma unless stated otherwise.

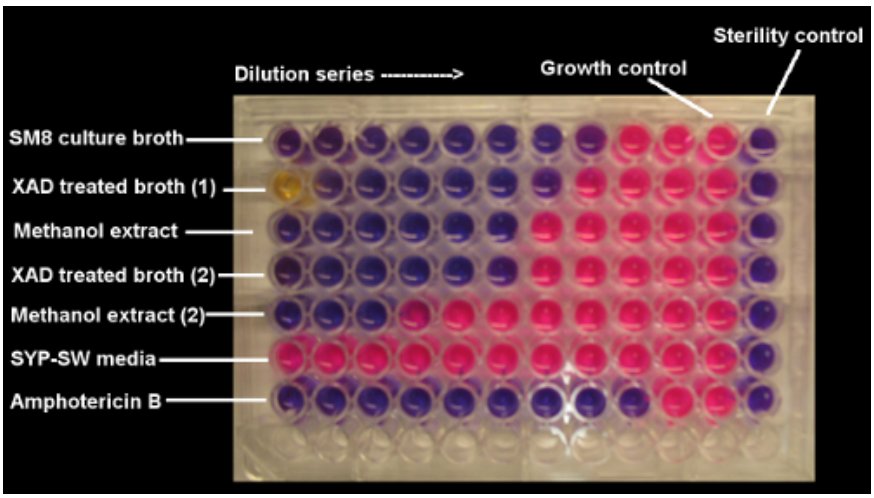


Figure 5.1 : Illustration of the NCCLS assay.

This figure illustrates the operation of the NCCLS assay. The test extracts (spent media, resin XAD treated broth and the methanol extract) from SM8 were tested in the plate assay against *C. glabrata*. The dye resazurin (blue) gets reduced to resorufin (pink) when the cells grow. The dye remains blue when there is inhibition of growth. As the extraction was done twice, both the extracts were tested hence labelled as (1 and 2). The media (SYP–SW) in which the strain was grown initially was the negative control and amphoterin B, an anti–fungal compound was the positive control. Both sterility and growth controls were also included in the assay.

5.3.5 Quorum sensing assay

The purified extract was dissolved in methanol at a concentration of 1 mg/ml. The test strains (*Chromobacterium violaceum* CV026, *Serratia marcescens* SP15 and SP19) were grown overnight in LB at 37°C (Poulter *et al.*, 2010); (McClean *et al.*, 1997). Positive (N–(hexanoy1)–L–

homo– serine lactone: HHL) and negative (methanol) controls were included in the assay plate. The test strains were streaked on to the LB to create a lawn of cells. 50 µl of the purified extract were added to the disc and the plate was incubated overnight at 37°C. The plates were examined for both stimulation (a violet halo in case of CV026, red halo in both SP15 and SP19) and antagonistic effect (a white halo in case of CV026, SP15 and SP19).

5.3.6 Calcineurin assay

SM8 crude extract and the purified fractions were screened in a method developed to detect inhibitors for the calcineurin pathway in yeast. The fractions that were purified were tested for the anti–calcineurin assay. The method is described in detail in Chapter 4.

5.3.7 Purification techniques

Various purification strategies were followed for the identification of the bioactives. Initially, the work was carried out at the Department of Microbiology and Pharmacy at University College Cork but as this strategy was unable to generate sufficiently pure compounds, subsequent work was carried out at the University of Strathclyde, Scotland.

5.3.7.1 Thin layer Chromatography (TLC)

Extracts were tested on a normal phase TLC silica plate. 5 µl of the extract was added to the plate and dried thoroughly. Meanwhile, the TLC chamber was saturated with the solvent system that varies each time depending on the mobility of the extracts. The plate was then allowed to run in the solvent system until it reaches three quarters of the plate. The solvent front was noted and the plate was dried thoroughly. It was then sprayed with the p–anisaldehyde–sulphuric acid, for the detection of lipids, sugars, phenols, terpenes and steroids (Serralheiro & Quinta, 1986). The plate was heated to 105°C until the spots are visible.

5.3.7.2 Medium Pressure Liquid Chromatography (MPLC)

Crude extract was fractionated using a VersaPak C18 (Spherical) 23×53 mm (15 g) column using methanol and water as the mobile phase (Ishihara *et al.*, 2000). The Medium Pressure Liquid Chromatography instrument (pump manager C615, pump module C601) came from BUCHI, Switzerland, whereas the columns used and the column stand were purchased from VersaFlash/Supelco, Sigma Aldrich, Germany. The fraction collector (CF2) was from Spectrum Labs. The gradient was as follows: 0–5 min at 1% methanol, 5–35 min at 1–50% methanol, 35–40 min at 50% methanol, 40–65 min at 50–100% methanol. The fractions were collected every 15 sec in a test tube. A total of 264 fractions were collected. The column was then washed three times with 100% methanol, each washing performed for five minutes and collected in

a conical flask. The fractions were pooled by the minute for the antimicrobial assay. 400 µl from each of four test tubes were combined and dried to give an aliquot of the extract for one minute. The process was repeated for each of the 65 min. The pooled fractions were then dried, weighed and tested for anti–fungal and anti–bacterial activities using the NCCLS assay.

5.3.7.3 Sephadex purification

The crude extract that was prepared by growing the strain in oatmeal in artificial sea water was passed through Sephadex LH–20 that was prepared by soaking it overnight in methanol to allow it to swell. It was then poured into the column in continuous motion and allowed to settle and washed by carefully running more methanol through the column. The crude sample was dissolved in methanol and loaded on to the column. The flow rate was approximately 1 ml/15 min. The fractions were tested for antimicrobial activity using the NCCLS assay. The active fractions were subsequently pooled based on their activity and similarities in the TLC.

5.3.7.4 Silica column purification

Active fractions were also subjected to a conventional silica column purification with the following solvent systems: 95:5 hexane:ethyl acetate, 90:10 hexane:ethyl acetate, 80:20 hexane:ethyl acetate, and 50:50 hexane:ethyl acetate, followed by washing of the column with 70:30 dichloromethane:methanol, 50:50 acetone:methanol and 100% methanol. The fractions collected were pooled following TLC and antimicrobial testing was done using the NCCLS assay.

5.3.7.5 High–performance Liquid Chromatography (HPLC)

In some cases, the samples were separated using HPLC which was run in an X Bridge C18 5 µm 4.6×150 mm analytical Column attached to an X Bridge C18 5 µm 4.6 ×20 mm guard column with a gradient of 0–100%ACN (0.1% formic acid) for 0–25 min and 5 min in 100% ACN (0.1% formic acid) at a flow rate of 1 ml/min. The samples were collected, concentrated and dissolved in 100 µl of methanol and tested using the NCCLS assay. The active fractions were further purified using a preparative C18 column with a gradient of 0–100% ACN (0.1% formic acid) for 0–25 min and 5 min in 100% ACN (0.1% formic acid) at a flow rate of 20 ml/min. The samples were collected, concentrated and dissolved in 1ml of methanol and tested using the NCCLS assay. This work was carried out in the Microbiology and Pharmacy Department at the University College Cork. The samples that were scaled up and extracted were analysed by low resolution LCMS. The samples were run using the LCQ Deca using the Column: ACE 5 C18 75×3.0 mm with the mobile phase being 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.4 ml/min. The gradient was as follows: 0–30 min

at 10%–100% B, 30–35 min at 100% B, 35–36 min 100%–10% B, 36–40 10% B (equilibration). The fractions were then pooled based on the similarities of their chromatograms. The separation of the compounds and the analysis by LCMS was carried out at the University of Strathclyde, Scotland.

5.3.7.6 High–resolution Mass Spectrometry (HRMS) and NMR

The samples were run on the Ultimate 3000 Exactive (Thermo Scientific, Germany) using the column: ACE 5 C18 75×3.0 mm (Highchrom Ltd, UK) with the mobile phase being (A) 0.1% formic acid in water , (B) 0.1% formic acid in acetonitrile and an injection volume of 10 µl. The gradient used was as follows: 0–30 min at 10–100% B, 30–35 min at 100% B, 35–36 min at 100%–10% B, 36–40 min at 10% B (equilibration). The flow rate was 0.3 ml/min. This was performed on the active fractions following which MZMine 2.8 was used to process the data, after which the data was run through the AntiMarin database to identify any known compounds (`http://www.chem.canterbury.ac.nz/marinlit/marinlit.shtml`). The samples were dissolved in 750 µl of deuterated DMSO and subjected to a ^{1}H NMR analysis. The samples were then lyophilized and redissolved in enough 50:50 methanol (HPLC):pure–grade water to give a final concentration of 1 mg/ml. The NMR used was the Jeol LA–400 FT–NMR spectrometer system with an AS400 magnet at 400MHz and the fractions were dissolved in DMSO. The purification and the NMR was carried out in University of Strathclyde, Scotland.

5.3.8 Collaboration between University College Cork and University of - Strathclyde

The following was the work done in UCC and University of Strathclyde, Scotland:

- UCC: Culture grown and crude extraction.
- University of Strathclyde: Purification, LC–MS and NMR analysis.

The collaboration is represented in Figure 5.2.

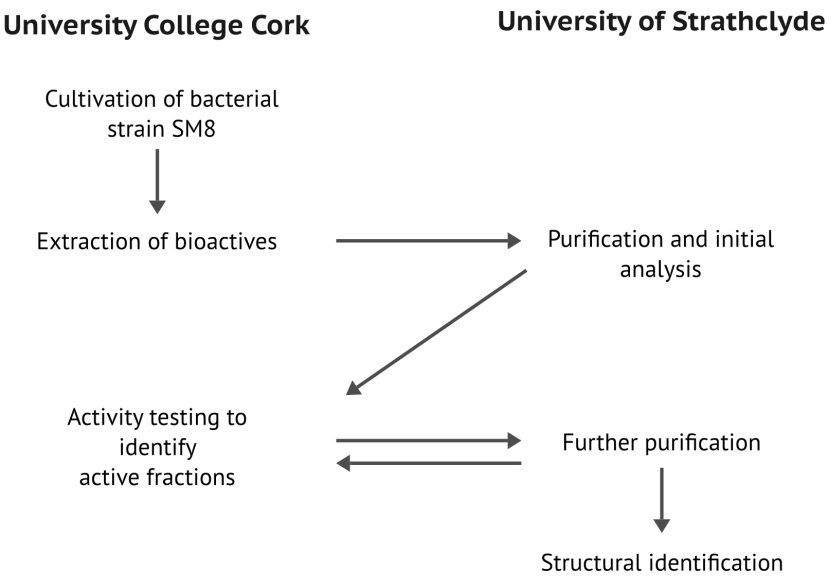


Figure 5.2 : Schematic representation of the collaboration between UCC and Strathclyde University.

The cultivation, extraction and activity testing was done in UCC and the characterisation of the compounds was done at University of Strathclyde, Scotland.

5.4 Results

5.4.1 Screening the strains for antimicrobial activity

The bacterial strains that were cultured from *Haliclona simulans* were screened for anti–fungal activity (Kennedy *et al.*, 2009). 120 strains were initially screened for anti–fungal activity with all the five test strains using a deferred antagonism assay and it was observed that there were 25 strains that had anti–fungal activity. The strains were then grown in a liquid culture to test for the anti–fungal activity using the well–diffusion assay. There were 17 strains that showed anti–fungal activity in well–diffusion assay and the anti–fungal compound was extracted from these strains using methanol after it was incubated with the amberlite XAD–16 resin. Several studies have shown that organic extraction has been effective for the isolation of bioactives (Kjer *et al.*, 2010). It was observed that only from 8 strains SM6, SM8, SM12, SM15, SM17 (*Streptomyces* spp.), 18A (*Halomonas* sp.), PA2 and PA4 (*Pseudoalteromonas* spp.) the activity was extractable in the organic phase whereas for the other strains SM1, SM2, SM3, SM4, SM7, SM9 (*Streptomyces* spp.) and MMA7 (*Bacillus* sp.) the activity remained in the broth. There were 2 strains SM5 and SM14 (*Streptomyces* spp.) that did not show any activity (Figure 5.3).

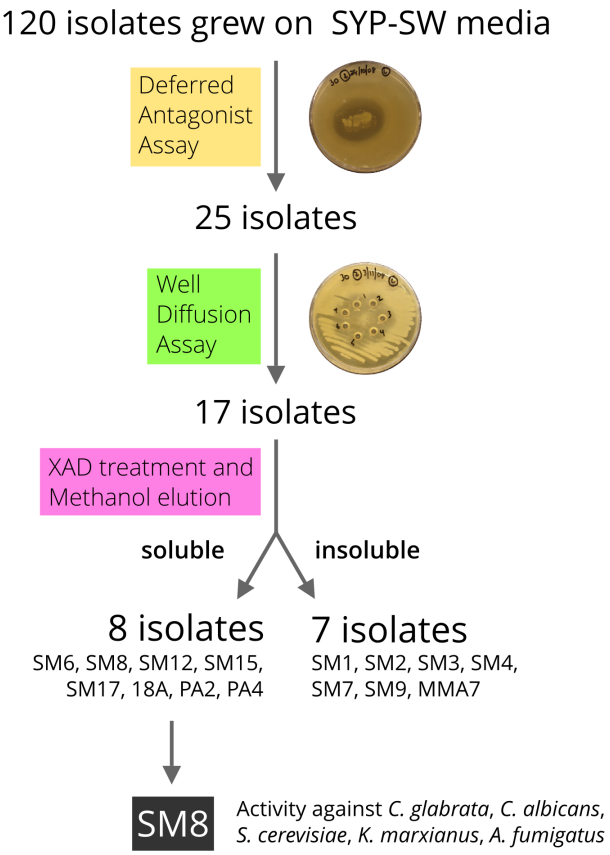


Figure 5.3 : Screening the bacterial isolates for anti–fungal activity from *Haliclona simulans*.

The isolates from *Haliclona simulans* were screened for anti–fungal activities using the overlay assay that shows a zone of inhibition when incubated at 28°C overnight. The putative isolates were grown in a liquid media for the production of secondary metabolites. The supernatant collected were tested and a zone of inhibition indicates that the strain is producing an anti–fungal metabolite. The positive isolates were cultivated in the liquid media and an organic extraction was done and it was detected that the activity was extractable for 8 isolates. SM8 strain showed activity against all fungal strains tested.

It should be noted that eleven isolates that secreted anti–fungal activity were *Streptomyces* sp. and in five of the eight isolates the activity was extracted by the organic solvent (Figure 5.4). This genus is known to produce bioactives and so was of interest to us (Dharmaraj & Sumantha, 2009). As the isolate SM8 grew well and showed consistent activity, it was selected for further study. The strain was then tested against bacterial strains such as *E. coli*, *B. subtilis*, *S. aureus* and *P. aeruginosa* (data not shown). It was observed that it was active against *B. subtilis* and *P. aeruginosa*. This suggested that the strain has a broad spectrum of activities.

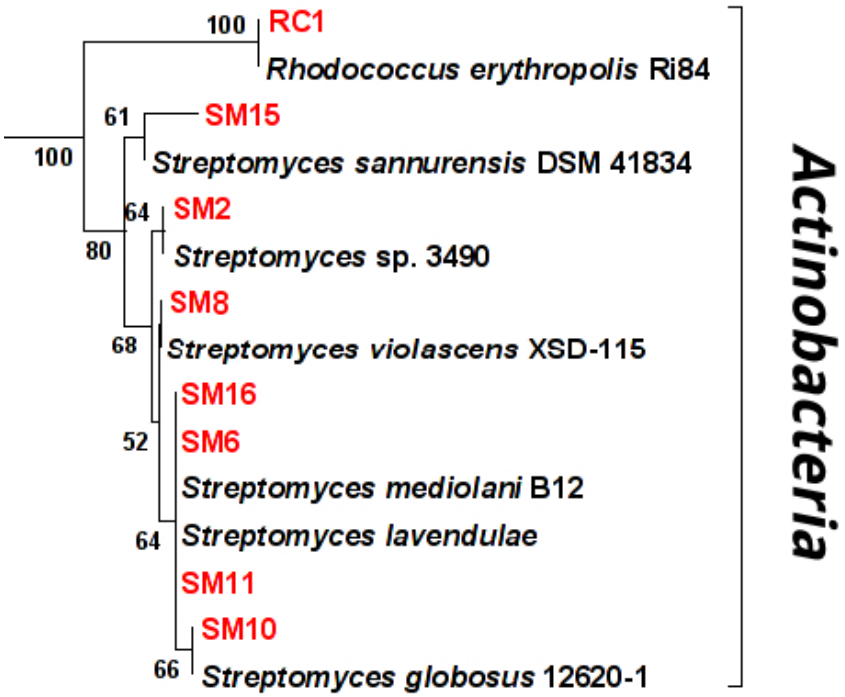


Figure 5.4 : Phylogenetic tree of the marine actinobacteria isolated from *Haliclona simulans*.

Adapted from (Kennedy *et al.*, 2009).

5.4.2 Purification and characterisation of antimicrobial compounds

Different purification strategies were employed to identify bioactive molecules from SM8. Initially, all analyses was in–house between Department of Microbiology and Pharmacy, subsequently a collaboration at University of Strathclyde, Scotland was established. The purification strategies are summarised in Figure 5.5 and described in detail below.

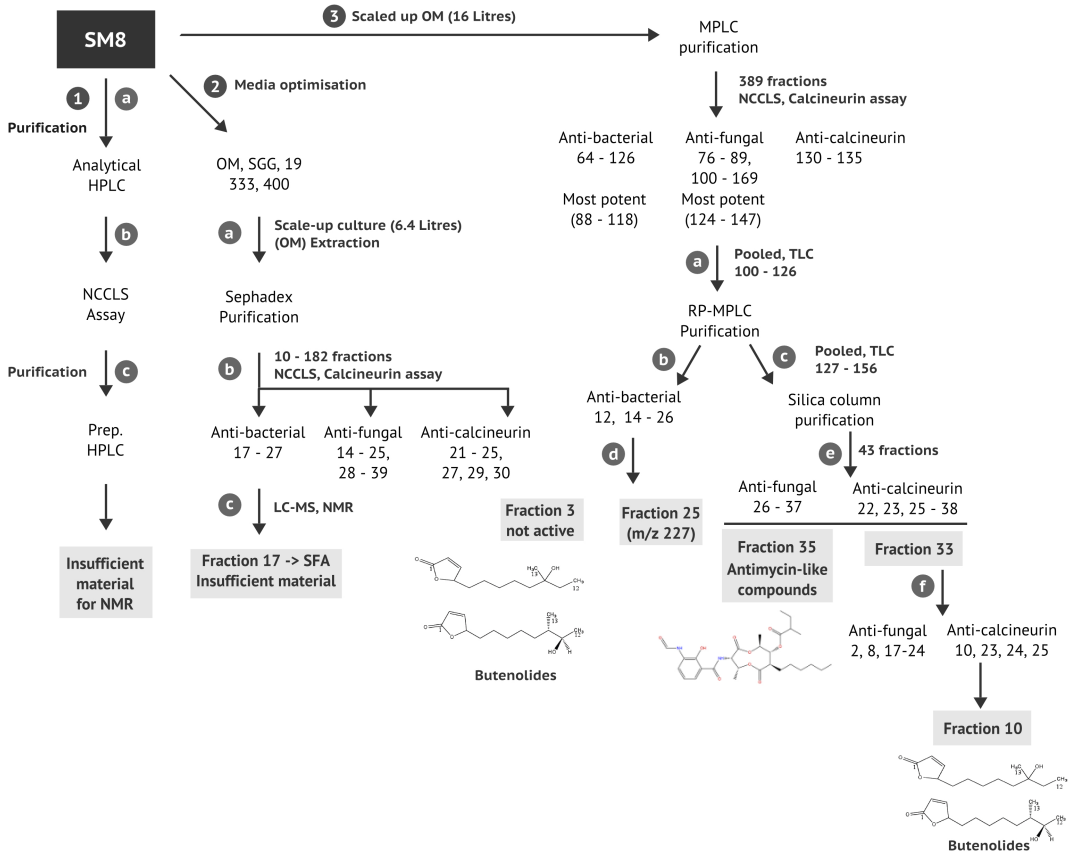


Figure 5.5 : Summary of the strategies involved in the identification of the metabolites in SM8.

The strain was grown in SYP–SW media initially and purified by preparative HPLC. As the material was insufficient for NMR, the media was optimised for maximal production of the metabolites. The culture was grown in the oatmeal media with artificial seawater and was scaled to 6.4 l. After sephadex purification, the active fractions were tested in the NCCLS assay for antimicrobial activity and calcineurin assay described in Chapter 4. One of the active anti–bacterial fractions (Fraction 17) was analysed by NMR and the fraction was identified as a hydroxylated saturated fatty acid. Due to insufficient material, the culture was scaled to 16 l and the extract was purified by MPLC. The fractions collected were tested for both antimicrobial and calcineurin assay. The active fractions were pooled based on relative mobilities in TLC and RP–MPLC purification was carried out further to determine the nature of the active metabolite. Fraction 25 that showed anti–bacterial activity was identified as a compound having m/z 227 and fraction 3 that was not active was identified as butenolides by NMR. The anti–fungal fractions were pooled based on relative mobilities in TLC and was purified by silica–column. The fractions were tested for anti–fungal activity and calcineurin assay. By NMR, one of the active anti–fungal fractions (Fraction 35) showed antimycin–like compounds. The butenolides were identified in Fraction 10 of the anti–calcineurin fractions.

5.4.2.1 Strategy 1: Analytical HPLC

The SM8 culture was scaled up initially in 6.4 l of SYP–SW media and the purification was done by analytical and preparative HPLC (Step 1). After several rounds of purification and also assaying the active fractions by the NCCLS assay for anti–fungal activity against *C. albicans*, the production of the compound was insufficient for determining the structure of the molecule. Hence a different strategy had to be followed so that metabolites can be produced enough to predict the structure of the compounds.

5.4.2.2 Strategy 2 : Sephadex Purification

The media (SYP–SW) that the strain was initially grown on did not produce enough for the structural elucidation and hence the media had to be optimised. It has been observed in several studies that the variation in the carbon and nitrogen sources in the media can influence the production of the secondary metabolites (Haritha *et al.*, 2012). Goodfellow and Fiedler in their studies have recently optimised several media for the cultivation of the Actinobacteria (Goodfellow & Fiedler, 2010). Hence the strain was grown in 5 different media and it was observed that the oatmeal media with the artificial seawater produced 3 fold more of the active compound when tested in the NCCLS assay for anti–fungal activity against *C. albicans*. It was initially scaled to 6.4 l and the active compound was extracted as mentioned in Section 5.3.5.3. The crude extract was tested by the NCCLS for both anti–fungal against *C. albicans* and anti–bacterial activity against *B. subtilis* (data not shown). The crude extract of about 343.7 mg was purified using Sephadex column (Step 2b) and the fractions were tested for anti–bacterial, anti–fungal and anti–calcineurin activities. The active fractions (Step 2c) were then subjected to LCMS and NMR and it was observed that one of the anti–bacterial fraction was a hydroxylated saturated fatty acid (Figure 5.6). Due to the insufficient material, the structure could not be elucidated.

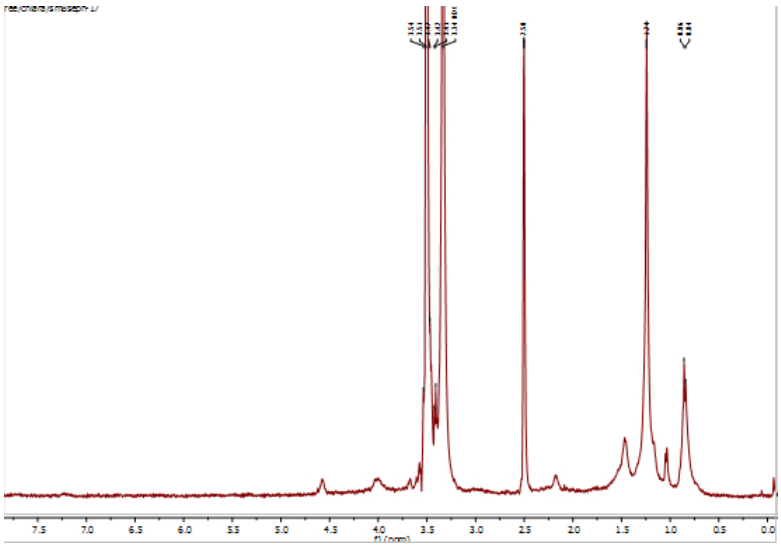


Figure 5.6 : NMR of the saturated hydroxylated fatty acid

^{1}H NMR of Fraction 17. The peak at 0.86 is the terminal methyl group. The peak at 1.24 represents the –CH2– groups. The peak at 2.5 is the solvent peak, DMSO and that at 3.34 is a water peak. The peak at 3.51 represents the hydrogens attached to carbons bearing hydroxyl groups. The peak at 2.18 represents the CH_2 attached to the carboxylic acid carbon.

5.4.2.3 Strategy 3 : MPLC Purification

The culture of SM8 was grown in 16 l of OM in artificial seawater media (Step 3) and the crude extract that weighed about 810.3 mg was sent for the purification and the identification of the metabolites. Initially the crude extract was purified using Sephadex LH20 using methanol as the mobile phase with a flow rate of 1 ml for every 15 min. 1 mg of each of these fractions were tested by the NCCLS for both anti–fungal and anti–bacterial activities. SM8 crude extract had shown calcineurin inhibition in the assay described in detail in Chapter 4. Hence the purified fractions were also tested for calcineurin inhibition. The active fractions were pooled based on their relative mobilities and the colour observed in TLC. Fractions 110–126 were pooled and taken for further analysis as it showed all three types of activity and also had the greatest quantity (170.9 mg). Hence these were separated further by reverse–phase MPLC using a C18 column with water and methanol as the mobile phase and a flow rate of 15 ml/min. The fractions were pooled based on the relative mobilities and the colour observed in TLC and tested for antimicrobial activities. Anti–fungal activity and anti–calcineurin activity was absent.

High–resolution Liquid Chromatography Mass Spectroscopy (HRLCMS) was performed on the active fractions (Step 3d) and the data was processed using MZMine 2.8. The data was then run through the AntiMarin database to identify any known compounds. Fraction 25 that had mild anti–bacterial activity against *B. subtilis* showed m/z 227 that was similar to medelamine B. 2D NMR was done to confirm the structure but the spectra did not match medelamine B

and so it could be an isomer or related compound. Due to the insufficient material, further analysis was not possible.

Fraction 3 which was relatively pure (Step 3d) though not active against *B. subtilis* was analysed for NMR. This resulted in the identification of two known furanone isomers. The chemical formula was $C_{13}H_{22}O_3$ and the exact mass was deduced as 226.16. The structures were also confirmed by 2D NMR experiments. The ROSEY spectrum aided in ascertaining the relative stereochemistry of the second compound. Recent studies have reported these butenolides as quorum sensing molecules (Husain *et al.*, 2010) ; (Chernin *et al.*, 2011). These furanones were then tested for quorum sensing assays as mentioned in Section 5.3.6. The quorum sensing assay did not show any stimulation of antagonistic effects in any of the test strains indicating that the butenolides could be inactive. Hence the biological property of these furanones (butenolides) were unknown.

In parallel, the anti–fungal fractions (127–156) that were obtained from the Sephadex separation were purified using a conventional silica column (Step 4e). It was observed that 43 fractions were active when tested (Table 5.1).

Table 5.1 : Fractions exhibiting anti–fungal and anti–calcineurin activity after MPLC purification.

Fraction number 33 is highlighted in bold as it weighs the most and was fractionated further to identify the metabolite.

Fraction Nos.	Weight (mg)	Anti–fungal activity (*C. albicans*)	Anti–calcineurin activity
		Specific activity (mg/ml)	
23	7	–	+
25	3	–	++
26	2.2	0.00625	+++
27	2.6	0.003125	++
28	6.2	0.00625	+
29	3.5	0.00625	+
30	6.5	0.0125	++
31	2	0.025	+
32	2.2	0.0125	+
33	**46.2**	**0.025**	**+**
34	10.7	0.025	+
35	2.3	0.025	+
36	3.5	0.05	+
37	12.4	0.05	+
38	6.6	–	+

Fraction 35 was run through LCMS (as it was relatively pure) and was identified as antimycin

by the AntiMarin database. Hence a comparison was done to the standard Antimycin A that detects four isomers of antimycins (A1–A4) by NMR. The sample showed the presence of compounds having similar m/z, indicating that the sample could possibly be antimycin A derivatives. However, the retention times were different indicating that the compounds may just be the isomers of antimycins. The total ion chromatograms after LCMS shows clearly that most of the compounds in fraction 35 elute within 25 min whereas the standard Antimycin A has retention times between 20 and 30 min (Figure 5.7). The fragmentation data obtained from LCMS from the standard showed the most common fragments of m/z 237 and 265. However, these were not observed in the sample. This data suggests that the anti–fungal metabolite(s) were antimycin analogues but it is unclear if the antimycins identified in SM8 are known or unknown. Due to the insufficient material, the structural elucidation of the antimycin was not possible.

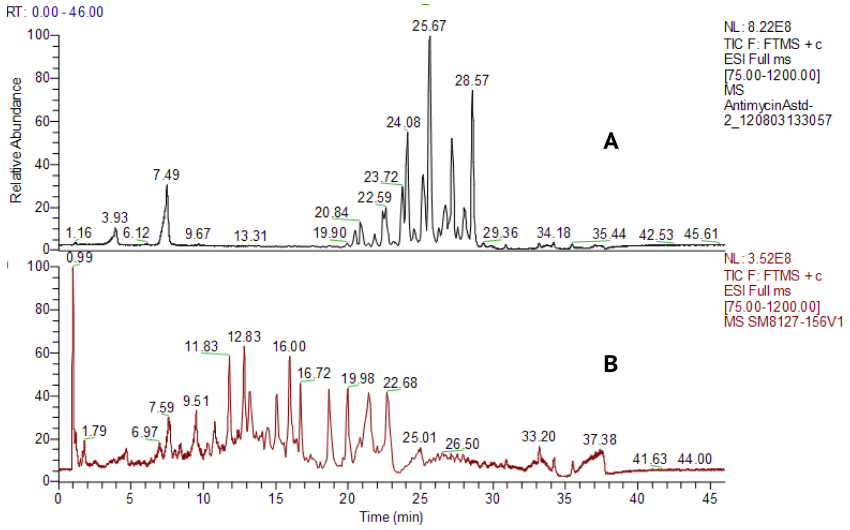


Figure 5.7 : Total Ion Chromatograms of Antimycin A standard (A) and fraction 35 from SM8 sample (B).

The retention times of the antimycin A derivatives evidently elute between 20 and 30 min whereas most compounds in the sample elute before 25 minutes.

Among the anti–fungal fractions, fraction 33 weighed 46.2 mg and was purified using the Biotage Flash Purification system as a mobile phase of hexane (A) and ethyl acetate (B) with a flow rate of 25 ml/min. After fractionation (Step 3f), the resulting fractions were pooled on the basis of the relative mobilities and the colour on the TLC. The fractions were then tested for both anti–fungal and anti–calcineurin activity. It was observed that fractions 2 –8 and 17–24 showed anti–fungal activity in the NCCLS (Table 5.2). It should be noted that the fractions 20–28 were the eluates collected while washing the column with different organic solvents. Both anti–fungal and anti–calcineurin activities were present in those eluates. Hence a fraction of the compound was not bound to the column. Further work has to be done to identify the metabolite(s) causing anti–fungal activity.

Table 5.2 : Anti–fungal and anti–calcineurin fractions obtained after purification of fraction 33.

Fraction Nos.	Anti–fungal activity *(C. albicans)*	Anti–calcineurin activity
	Specific activity (mg/ml)	
2	0.0125	–
3	0.025	–
4	0.05	–
5	0.05	–
6	0.025	–
7	0.05	–
8	0.05	–
9	–	–
10	–	+
11	–	–
12	–	–
17	0.05	–
18	0.05	–
19	0.05	–
20	0.05	–
21	0.025	–
22	0.05	–
23	0.05	+
24	0.05	+
25	–	+

Fraction 10 that showed only anti–calcineurin activity was relatively pure and hence ^{1}H NMR was done to determine the structure. The same furanone isomers that had been identified in Step 3d was obtained in fraction 10 (Figure 5.9 a, b). The calcineurin assay was performed as discussed in Chapter 4. It was observed from the assay that four of the fractions showed calcineurin inhibition (Figure 5.8). The inhibition observed in lanes 6–8 is from the washing of the column with different concentration of ethylacetate and methanol. Fraction 10 that was relatively pure showed calcineurin inhibition is represented in lane 4. This was the only fraction from the SM8 fraction that showed only calcineurin inhibition. From the data (lanes 3 and 5), it should be noted that the fractions 9 and 11 did not show any calcineurin inhibition indicating that the anti–calcineurin is not spread out unlike the anti–fungal fractions (Table 5.2).

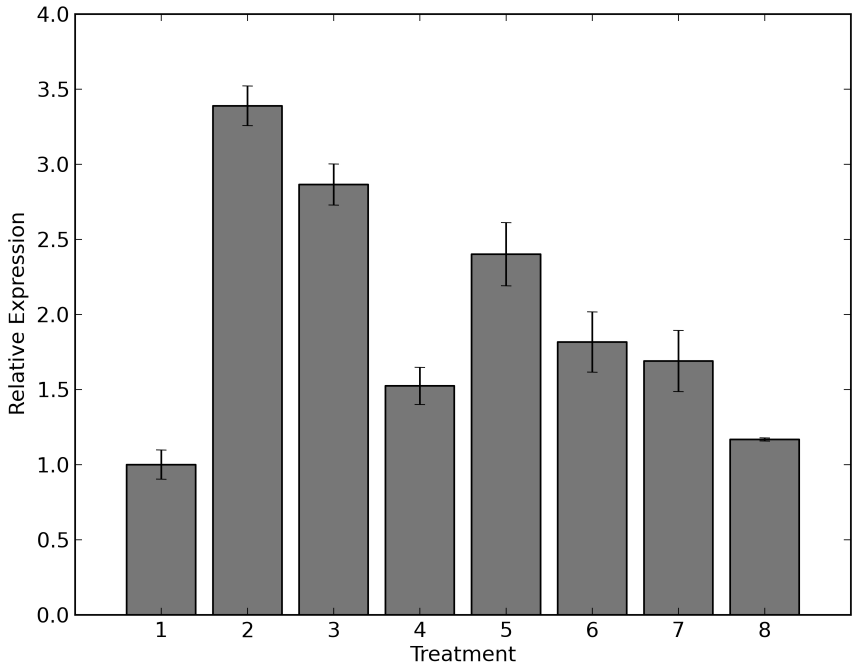
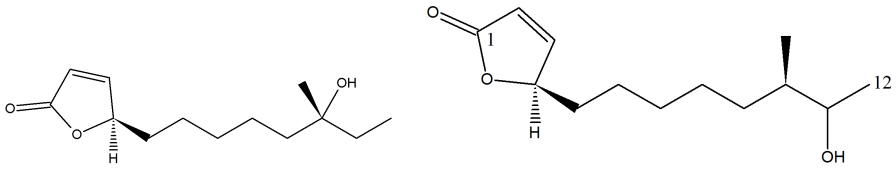


Figure 5.8 : Bacterial extract of purified SM8 from fraction 33 showing calcineurin inhibition.

The assay was as described in Figure 4.1 of Chapter 4, with methanol as controls. With the exception of lane 1 (negative control), all treatments included TAPS alkaline shock and all treatments included the same solvent (10% methanol). Fractions 9 and 11 are represented in lanes 3 and 5 respectively, and data from the four positive fractions are shown in lanes 4 and 6–8. Fractions are denoted by numbers. Lane 1, negative control, no stimulation; lane 2, positive control; lane 3, fraction 9; lane 4, fraction 10; lane 5, fraction 11; lane 6, wash1; lane 7, wash 2; lane 8,wash 3. It is seen that the relative expression in lane 3 and 5, is the same as the positive control (lane 2) whereas relative expression in lanes 4 and 6–8 is more similar to that of the negative control (lane 1). Assays were carried out in triplicate.



a **b**

Figure 5.9 : Structure of butenolides.

Two furanone isomers were obtained in fraction 10 :

a) 4,10–dihydroxy–10–methyl–dodec–2–en–1,4–olide.

b) 4,11–dihydroxy–10–methyldodec–2–en–1,4–olide.

From the NMR spectra, the signals at 0.90 ppm and 1.00 ppm were used to estimate the relative amounts in each butenolide. The red singlet peak at around 0.99 ppm was much taller than

that of the teal peak, but the two smaller peaks beside it (the doublet at around 0.95 and 0.96 ppm) were about the same. The methyl signals are the only resonance that could differentiate both the butenolides. These signals represent the methyl at position 13 in isomer 1 (Figure 5.8a) which was a singlet and the methyl at position 12 in isomer 2 (Figure 5.8b). Those signals were chosen because they were not overlapping when compared to the methyl at position 12 in isomer 1 which was a triplet and the methyl at position 13 in isomer 2 which was a doublet. These latter signals found between 0.80 ppm and 0.70 ppm were overlapping and hence will not be possible to integrate separately (Figure 5.10).

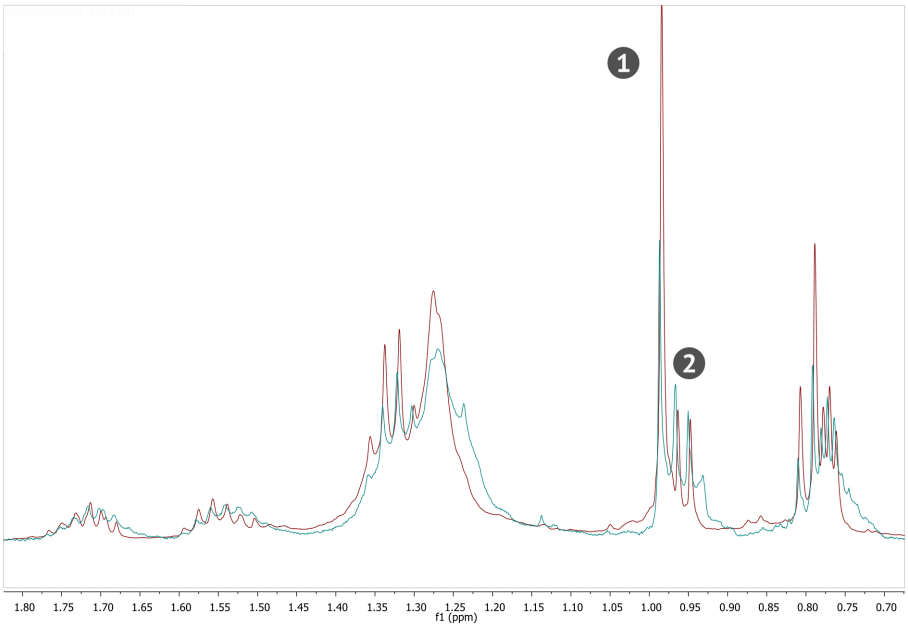


Figure 5.10 : ^{1}H spectra fraction 3 (inactive) is the red spectrum whereas fraction 10 (active) is the teal–coloured spectrum.

The NMR spectra was zoomed in between signals 0.70 ppm and 1.80 ppm. The only difference between the two fractions was the ratio between the two butenolides. Both the isomers were present in almost the same quantity in fraction 10. A certain number of protons were assigned to the peak chosen for the intergration. From the intergration of the peaks, the percentage of the furanones present in each of the fraction were calculated.

As ^{1}H NMR is quantitative, the integral peaks between the two isomers were calculated. The integration was based on the area under the peak and it also represents the number of protons within a peak (Table 5.3).

Table 5.3 : The percentage of the isomers present in each of the fractions of the butenolides are tabulated.

	Integration 1	Integration 2	Isomer 1	Isomer 2
Fraction 3	1.00	0.40	71.43%	28.57%
Fraction 10	1.00	1.26	44.25%	55.75%

1 mg of the fractions were tested. Fraction 3 was found to be inactive whereas fraction 10 displayed anti–calcineurin activity. This activity was dependent on the concentration of isomer 2 when compared to isomer 1 in fraction 10. Further work will involve separating these two isomers so that the activity of the isomers can be validated.

5.5 Discussion

5.5.1 Purification of bioactive metabolites : strategies and challenges

Different purification strategies were employed to identify the bioactives as described in Figure 5.5. Some of the challenges that had to be faced during the development of the extraction and the purification strategies was that sample material usually obtained during the crude extraction contains a mixture of other compounds and the compound of interest present in them was minimal. As a result, structural elucidation of some of the active compounds was not possible. The supply problem has always been the bottleneck in the natural product drug discovery process. The purification strategies requires extensive optimisation to facilitate chemical characterisation of compounds (Penesyan *et al.*, 2010). Recently, novel techniques have been employed for rapid detection of compounds. One study has used an ultra–performance liquid chromatography (UPLC) where the data can be obtained in $^{1}/_{10}{}^{\text{th}}$ of the time and the sample used would be less when compared to the traditional HPLC or other purification strategies (Rowe *et al.*, 2010). Another technique that was developed recently was Desorption Electrospray Ionisation Mass Spectrometry (DESI–MS) that allows rapid detection of compounds with minimal effort on data analysis and sample preparation (Nielen *et al.*, 2009). Scientists from China have recently developed an alternative to chromatographic methods for separating bioactive compounds in large scale. They employed a high–performance separation technique to separate sparingly aqua–/lipo–soluble bioactive compounds using an ionic liquid–based biphasic system (Cao *et al.*, 2012).

5.5.2 Identification of bioactives

This study identified one particular *Streptomycete*, designated SM8 that synthesised several distinct bioactive metabolites exhibiting anti–bacterial, anti–fungal and anti–calcineurin activities.

5.5.2.1 Anti–bacterial metabolites

The anti–bacterial activity in SM8 was contributed by two distinct class of compounds: a saturated, hydroxylated fatty acid and an unknown metabolite of m/z 227. The structural elucidation of both the compounds were not possible due to the insufficient material. There are reports of bioactive fatty acids that are promising as drug candidates (M. Pereira *et al.*,

2011); (Mohamad *et al.*, 2009). The fatty acid that has been identified so far from marine were unsaturated fatty acids. Though the structure of the fatty acid from SM8 could not be identified due to the supply problem, the NMR spectra reveals that it is a saturated hydroxylated fatty acid and this is the first report of a saturated fatty acid isolated from *Streptomyces* sp. associated with a marine sponge. The unknown bioactive still has to be characterised by employing a better purification strategy.

5.5.2.2 Anti–fungal bioactives

The strategies employed to identify anti–fungal metabolites has to be improved as the activity was spread out (Table 5.1, 5.2) and this could be due to the presence of more than one anti–fungal metabolite in the extract. The data suggests the presence of antimycins (Figure 5.11) in the extract and the diversity of antimycins could also be the source of the broad anti–fungal activity.

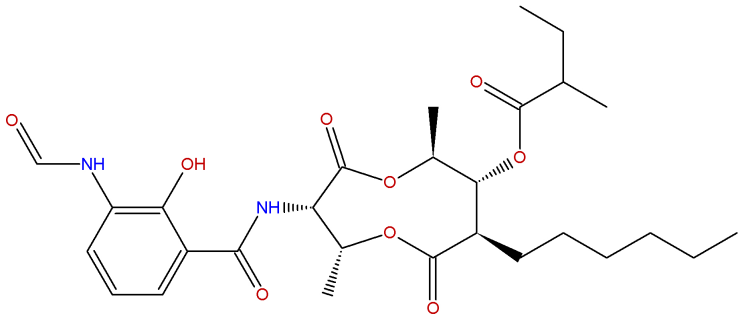


Figure 5.11 : Structure of Antimycin A1a.

Molecular weight: 548.625, Molecular formula: $C_{28}H_{40}N_2O_9$. This is a representation of one of the isomers of antimycin present in the standard that was used to compare with the SM8 extract.

Several antimycins have been discovered in recent years from various *Streptomyces* sp. The class of antimycins were initially discovered in soil *Streptomyces* sp. (Barrow *et al.*, 1997). Recent studies have isolated the initial class (A1–A4) of antimycins in addition to several other antimycins (A10–A16) from marine–related *Streptomyces* sp. (Hosotani *et al.*, 2005). Researchers in China have identified two new antimycins from the culture broth of a marine–derived *Streptomyces* sp. (Xu *et al.*, 2011). They also observed other classes of antimycins in the extracts such as A1a, A1b, A2a, A3a and A3b. Two antimycin A analogues were also identified recently from a marine–derived *Streptomyces lusitanus* (Han *et al.*, 2012). The diverse nature of antimycins suggests that in the present study, SM8 strain could be producing known antimycins or unknown ones as the fragmentation pattern and the retention time varied in the purified fraction when compared to that of the standard antimycins (A1–A4). Scientists from UK observed similar results in a *Streptomyces* sp. strain S4 that was a symbiont of a farming ant, *Acromyrmex octospinosus*. They found antimycin gene clusters and after the knockout, the anti–fungal activity was reduced but not abolished. Their genomic approach revealed clusters that could possibly produce multiple anti–fungals (Seipke *et al.*, 2011). Hence

a similar approach can be employed to detect the presence of other anti–fungals in SM8.

5.5.2.3 Anti–calcineurin compound

Anti–calcineurin activity identified in the SM8 strain was a butenolide (furanone) and this could be the first report of furanones exhibiting calcineurin inhibition in yeast (Figure 5.9). Earlier studies have reported these butenolides from a marine sediment (Mukku *et al.*, 2000). These butenolides have shown cytotoxic activity in recent studies (Olano *et al.*, 2009). There are no other activities reported to date with respect to these diastereomeric isomers. The butenolides that exhibited anti–calcineurin can further be analysed by synthesising and separating the furanones which can allow for a better understanding of the compounds. Scientists in Germany and Denmark have employed a new technique to isolate individual conformational isomers from a complex molecule using electrostatic fields at ultracold temperatures (Filsinger *et al.*, 2009).

5.5.3 Future directions

Dereplication process should be employed earlier in the stages of natural product identification. An Austrialian Biodiscovery company, Microbial Screening developed a software called COMET that compiles and analyses co–metabolite patterns in natural product mixtures. COMET analyses the data generated by DAD-HPLC and creates compact databases of the chromatograms and UV spectra. These databases can then be interrogated with flexible analytical tools, which enable rapid understanding of complex natural product extracts. This can be helpful for the recognition of novel co-metabolite patterns and also in the elimination of known metabolites producers and replicates early. Further analysis include cytotoxicity testing, identfying the mode of action and the genetic characterisation of the biosynthetic pathway of all the compounds identified in the marine *Streptomyces* strain should be pursued. The butenolides need to be further tested to identify specific effects and in this respect, a wide array of mutant strains and cell biology reporters available for yeast would be very useful in performing secondary screens and establishing mechanistic effects. This study has also yielded other metabolites that have not yet been characterised and may have potential as new therapeutic leads.

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Chapter 6

Genome Mining of a *Streptomyces* strain to identify novel bioactives[1]

6.1 Abstract

Genetic and genomic approaches were used to identify genes from *Streptomyces* strain SM8 that are potentially responsible for antimicrobial activities. The SM8 genome was sequenced from a fragment library using Roche 454 pyrosequencing resulting in 229,280 reads, giving approximately 13–fold coverage. These reads were quality filtered and assembled using the meta-assembler hosted by CAMERA 2.0 resulting in a draft genome of 534 contigs and a predicted genome of approximately 7.15 Mb. The draft genome was annotated by IMG/ER using the "Prodigal" pipeline. The SM8 genome contains a number of loci that are predicted to encode polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) as well as biosynthetic genes for terpenes and siderophores. Comparative genomic analysis with other *Streptomycetes* identified one PKS cluster, likely to direct synthesis of a candicidin-like molecule; one hybrid PKS/NRPS cluster for antimycin synthesis; two other PKS/NRPS hybrid clusters of unknown function; and four NRPS clusters of which one is predicted to be involved in gramicidin synthesis and the functions of the other clusters are as yet unknown. Analyses of mRNA levels revealed the expression of three hybrid clusters of which one is predicted to be involved in the synthesis of antimycin and the functions of others are unknown. There were an additional two NRPS clusters of which one is similar to gramicidin biosynthesis and the function of the other is as yet unknown. The Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AMPN00000000.

[1]Manuscript in preparation

6.2 Introduction

Streptomyces are known to produce bioactive metabolites that include antibiotics such as streptomycin, tetracycline and vancomycin anti–fungals such as nystatin and natamycin, immunosuppressants such as cyclospirin A and many more (Borodina *et al.*, 2005). They are ubiquitous in nature and have distinguishable characteristic features such as high G+C content, a large linear chromosomes and a complex life cycle.

The large and complex genomes of *Streptomyces* are believed to be related to their adaptability to different environmental conditions (van Wezel & McDowall, 2011). Secondary metabolism genes tend to be located at the distal ends of the chromosome arms whereas the central part of the chromosome contains a mix of primary and secondary metabolism genes (Chater, 2006).

The metabolites produced by this genus are quite diverse in their structure and function, for example antimycin, gramicidin, avenolide and many more. The genes for secondary metabolism pathways are arranged in defined genetic regions or clusters on the chromosome. These clusters are typically comprised of genes required for the synthesis of a specific metabolite including carbon chain assembly, modification, export, resistance and regulation.

The advent of whole-genome sequencing revealed that the number of secondary metabolism gene clusters in bacteria such as *Streptomyces* and in fungi outnumbers the known secondary metabolites produced by these microbes (Scherlach & Hertweck, 2009), as many of these clusters are apparently silent under standard conditions (cryptic clusters). The role of the secondary metabolites in these microbes remain unclear but many are believed to be produced as a means of chemical defence, as signalling molecules, as metal scavengers and some are believed to have a role in microbe–host interaction (Rimando *et al.*, 2007).

In *Streptomyces*, the diversity of the secondary metabolic pathways is enormous comprising PKS, NRPS, siderophores, ribosomal peptides and terpenes (Fu *et al.*, 2012). Genome mining approaches have made it possible to manipulate gene expression and alter the regulation of secondary metabolism clusters, for example overexpression of a regulatory gene flanking the NRPS genes enhanced the production of daptomycin in *Streptomyces roseosporus* (Yu, 2012).

In silico tools have helped researchers in the identification of various gene clusters in the *Streptomyces* genus. Some of the examples include antiSMASH that helps in the rapid identification, annotation and analysis of secondary metabolite clusters (Medema *et al.*, 2011), PKS/NRPS analyser that predicts the specificity of the domains (Bachmann & Ravel, 2009), SBSPKS that helps in the prediction of the domains and the structure of the metabolite (Anand *et al.*, 2010) and ClustScan, a semiautomatic annotation of modular PKS and prediction of novel structures (Starcevic *et al.*, 2008). The knowledge of the genome sequences of the producing strains may pave the way for the manipulation of the biosynthetic genes involved in the biosynthesis of some of these antibiotics, thereby allowing the biosynthesis of variants with novel properties against the current drug resistant clinical pathogens; using combinatorial based approaches. It should be noted however that not all classes of antibiotics can be

used for such manipulations. An example of one class of antibiotic which are suitable candidates for the combinatorial approach are the peptide–based antibiotics such as vancomycins, bacitracins which are synthesized on NRPS based assembly lines (Walsh, 2002).

Genome–based approaches have also revealed the presence of various cryptic gene clusters that could be of clinical importance in the future (Gomez-Escribano & Bibb, 2011); (Komatsu *et al.*, 2010). One of the best example is the revelation of an uncharacterized Type I polyketide synthase gene cluster (*cpk*) in *S. coelicolor* A3(2) showing anti-bacterial activity after the deletion of a regulatory gene within the *cpk* cluster (Gottelt *et al.*, 2010). Hence manipulating regulatory pathways can "awaken" silent gene clusters and lead to the discovery of novel antimicrobial activities. One of the advantages of the genome mining approach is the revelation of many silent clusters that can be activated to provide potential new compounds. It can also be employed by researchers to alter specific pathways for the increased production of bioactives (Bromann *et al.*, 2012).

The aim of this study was to identify gene clusters involved in secondary metabolism in *Streptomyces* SM8 and to study the expression of genes potentially involved in secondary metabolite production.

6.3 Materials and Methods

6.3.1 Extraction of genomic DNA

Streptomyces strain SM8 was isolated from the marine sponge *Haliclona simulans* as described previously (Kennedy *et al.*, 2009). The strain was grown in 50 ml of SYP–SW media in a 250 ml flask for three days at 28°C in a shaker incubator at 200 rpm and the genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Kieser *et al.*, 2000b). The mycelia was resuspended in 5 ml TE25S (25 mM EDTA, pH 8, 25 mM Tris-HCl, pH 8 and 0.3 M sucrose) and 100 µl of 2 mg lysozyme/ml and incubated for 1 h at 37°C. This was followed by the addition of 50 µl of 25 mg proteinase K/ml and 300 µl of 10% SDS and incubated for 1 h at 55°C with occasional mixing. This was followed by the addition of 1ml of 5 M NaCl and was mixed thoroughly after the addition of 0.65 ml CTAB/NaCl (10% CTAB in 0.7M NaCl) and incubated at 55°C for 10 min. This was cooled to 37°C and 5 ml of chloroform/isoamyl alcohol (24:1) was added and mixed for 30 min by inversion. The solution was centrifuged for 30 min at 12,074×g to precipitate the proteins. The supernatant was transferred to a fresh tube and the DNA was precipitated by the addition of 0.6 vol. of isopropanol. The DNA was pelleted by centrifugation at 18,643×g at 4°C for 30 min in Eppendorf 5417R. The DNA was then washed with 500 µl of 70% ethanol and centrifuged at 18,643×g at 4°C for 15 min in an Eppendorf 5417R centrifuge and the pellet was air-dried. The DNA was then resuspended in 1 ml of TE buffer (10mM Tris-HCl, pH 7.5 and 1mM EDTA, pH 8) at 55°C. This was followed by the addition of 1 µl of 10 µg RNase A/ml to the DNA and incubated at 37°C for 30 min. The DNA

was then recovered by adding $^{1}/_{10}{}^{th}$ volume of 3 M sodium acetate of pH 6.8 and 0.6 volumes of isopropanol. The DNA was recovered as described above. The pellet was resuspended in 1 ml of TE buffer. DNA concentration was measured using a Nanodrop spectrophotometer (ND1000, ThermoFisher Scientific). The concentration was 200 ng/µl and the $^{260}/_{280}$ ratio was found to be 1.8. DNA was analysed by agarose gel electrophoresis and the average fragment size was found to be ~40kb. The DNA was then sent to the University of Liverpool, Centre for Genomic Research (`http://www.liv.ac.uk/integrative-biology/facilities-and-services/centre-for-genomic-research/`) for genome sequencing.

6.3.2 Genome sequencing

The nucleotide sequence was generated from a fragment library using the GS FLX Titanium system (Roche) resulting in `229,280` reads and `94,668,678` bp, giving approximately `13`–fold coverage. Assembly was carried out by meta-assembler hosted by CAMERA and annotated by the IMG/ER using the Prodigal pipeline.

6.3.3 Bioinformatics tools

Secondary metabolite genes were identified using antiSMASH (Medema *et al.*, 2011) that rapidly identifies a whole range of known secondary metabolite compound classes (polyketides, non-ribosomal peptides, terpenes, aminoglycosides, aminocoumarins, indolocarbazoles, lantibiotics, bacteriocins, nucleosides, β–lactams, butyrolactones, siderophores, melanins and others). This online bioinformatics tool also aligns regions at the gene cluster level to their nearest relatives from a database containing all other known gene clusters. The contigs were used as queries to scan for functional similarities both in Clusters of Orthologous Groups of proteins (COGs) and Pfam databases in IMG/ER. The IMG/ER has about `88` COG proteins and `52` Protein families (Pfam) relating to secondary metabolism and synthesis, catabolism and transport. The protein sequences corresponding to the secondary metabolite genes were searched in BLASTp (Altschul *et al.*, 1990), a protein database hosted by National Centre for Biotechnology Information (NCBI) to identify any functional similarities to other species. A BLAST search was done using the DNA sequence of the contigs of the secondary metabolism gene clusters to identify the closest homologues. The contigs were then aligned using CodonCode Aligner (`http://www.codoncode.com/aligner/`) to the best matching homologue in the corresponding reference genome.

6.3.4 Gap closure of a hybrid cluster

PCR primers were designed to target the predicted gaps between the contigs using Primer3-plus (Untergasser *et al.*, 2007). Genomic DNA was used as a template to amplify the inter-contig regions and the products were then purified and sequenced by GATC Biotech (`http:`

//www.gatc-biotech.com/en/index.html). The sequences were edited by FinchTv and then verified by querying in BLASTx. The sequences were then realigned with the contig sequences and manually edited using the GeneQuest software (http://www.dnastar.com/t-sub-products-lasergene-genequest.aspx) and the ORF was predicted by GeneMark (Besemer & Borodovsky, 1999). The predicted proteins were analysed by BLASTp and the secondary metabolism genes were analysed by antiSMASH. The domains were analysed by the PKS/NRPS analyser (O. Bachmann & Ravel, 2009) and SBSPKS (Anand *et al.*, 2010).

The genome analysis was performed by Ciarán O'Brien, an M.Sc. in Bioinformatics student at University College Cork.

6.3.5 Total RNA isolation

The strain was grown in both the SYP-SW (10 g starch/l, 4 g yeast extract/l, 2 g peptone/l, 33.3 g Instant Ocean/l (Atkinson & Bingman, 1998)) and oatmeal media (OM) (20 g oatmeal/l, 33.3 g Instant Ocean/l) in 50 ml in a 250 ml flask for 12 days at 28°C in a shaker incubator at 200 rpm. The supernatant and the mycelia were collected every second day from day 2 to day 12 and stored at −80°C. To preserve RNA, 2 volumes of RNA protect (Qiagen) reagent were added to samples immediately after sampling, mixed and incubated for 5 min at room temperature (15–25°C). The mycelia was then collected by centrifugation at 5000×g for 15 min, then kept at -80°C for long term preservation. To obtain total RNA from the bacteria several enzymatic and mechanical disruption of cells were performed. Initially, the cell pellet from a 3 ml culture was subjected to 100 µl of 15 mg lysozyme/ml in TE buffer and 20 µl of 25 mg proteinase K/ml in water. The pellet was resuspended with the pipette several times and incubated at room temperature (15–25°C) for 10 min. This was followed by mechanical disruption with the bead beater for 7 min using 3 mm glass beads. RNA was then purified using the Qiagen RNeasy Mini Kit. Total RNA was measured using a Nanodrop spectrophotometer (ND1000, ThermoFisher Scientific). Purified RNA was stored at −80°C until further use.

6.3.6 Preparation of cDNA

To remove residual genomic DNA, RNA concentration of 1 µg was treated with 1 unit of DNase I (37°C for 30 min) followed by the addition of 1 µl of 50 mM EDTA in a 50 µl reaction and incubated at 65°C for 10 min. 1 µl of RiboLock™ RNase Inhibitor at 1 u/µl was included in the reaction mixture to prevent RNA degradation. DNase treated RNA was measured using a Nanodrop spectrophotometer (ND1000, ThermoFisher Scientific). cDNA was synthesised by adding 4 µl of 5X reaction mix (reaction buffer, dNTPs, oligo $(dT)_{18}$ and random hexamer primers) (Fermentas), 2 µl of the Maxima® Enzyme Mix (Maxima® Reverse Transcriptase and Ribolock™ RNase Inhibitor) to 9 µl of the template RNA (~0.5 µg). The total volume was made up to 20 µl with nuclease-free water. RT–PCR was done with the following conditions: 10 min at 25°C followed by 30 min at 50°C and 15 min at 60°C. The reaction was terminated by heat-

ing at 85°C for 5 min. To confirm that the cDNA was not contaminated with genomic DNA, a PCR reaction using primers 27F (5´ AGAGTTTGATCCTGGCTCAG 3´), 1492R (5' GGTTACCTTGTTACGACTT 3') targeting the 16S rRNA gene was performed for both DNase treated RNA and cDNA (Turner *et al.*, 1999). The kits and the reagents were purchased from Fermentas (Wiame *et al.*, 2000); (Fleige & Pfaffl, 2006); (Nolan *et al.*, 2006).

6.3.7 Gene–expression studies

To analyse expression of putative secondary metabolism genes, individual genes from each cluster were chosen for analysis. Firstly the annotation of the secondary metabolism genes was checked manually to verify the start and the stop codons. Nine genes (Table 6.1), predicted to encode biosynthetic enzymes were selected and primers were designed to amplify a region of 500–1000 bp from each of these using Primer3Plus (Untergasser *et al.*, 2007). The specificity of the PCR was checked using genomic DNA. PCR was performed with a total volume of 50 µl containing sterile water, 5 µl of 1 X Taq buffer, 5 µl of 2 mM dNTPs, template DNA/cDNA (5 µl), Taq DNA polymerase (0.75 U) (Fermentas), and 5 µl of the primers (Stock concentration of 10 µM each). The conditions were as follows: initial denaturation (95°C for 5 min), followed by 36 cycles of denaturation (94°C for 30 sec), primer annealing for 60°C for 30 sec (exceptions being candicidin and unknown NRPS2 in which the annealing temperature was 59°C) and primer extension (72°C for 2 min), completed with a final primer extension step (72°C for 10 min). The PCR products were sent for sequencing to GATC Biotech (http://www.gatc-biotech.com/en/index.html).

Table 6.1 : Primers designed for amplification of genes involved in secondary metabolism clusters in *Streptomyces* SM8.

The primers were designed for specific regions in the contigs and the predicted size of the PCR product is listed in the table. The contig numbers and the gene ID are assigned by the IMG/ER Prodigal pipeline.

Predicted cluster	Contig	Gene ID	Primer sequences (5´–3´)	Predicted metabolite	Predicted size (bp)
Candicidin	183	2512070663	FP–CGAGGAAGCCGGACCAGT RP–CGTGCGCCGAGGTACT	Candicidin	559
Gramicidin	326	2512072684	FP–GACGAGCAGCTGACCTACG RP–AGCAGTTCGGTGACGCTGT	Gramicidin	560
Unknown NRPS1	113	2512069548	FP–CTTCCAGGTCATGCTCAACC RP–GACGGGAGTGGGTTCCAG	Unknown	640
Unknown NRPS2	223	2512071193	FP–CGGCGAGCTGTACCTGAC RP–GACCGGGATGTGGTACGAG	Unknown	853
Unknown NRPS3	6	2512067507	FP–GCGTACACCGTCGTGGAG RP–GGGGCCGTAGTGGTTGAC	Unknown	669
Unknown Hybrid1	271	2512071878	FP–GTGGTGTTGTGCGGGTTAC RP–CGCAGAGCAGTGTCCTGA	Unknown	670
Antimycin	406	2512073559	FP–ACACCGCCCTCTTCGAGAC RP–GATGTCCGAGGCCGTACC	Antimycin	755
Unknown Hybrid2	195	2512070890	FP–AGTTCGAACCGGCCTTCTT RP–CGGACCAGGGAGATCTGG	Unknown	873
Unknown Hybrid3	111	2512069504	FP–CTGCTCGACCTCCTCCAC RP–GGTCACGCACTGGTCCTT	Unknown	661

6.3.8 Antimicrobial assay

The antimicrobial assays were performed as described previously in Chapter 5, Section 5.3.4.

6.3.9 Calcineurin assay

The calcineurin assay was performed on the SM8 fractions as described previously in Chapter 4, Section 4.3.2.

6.3.10 Construction of antimycin knockout strains

To inactivate the antimycin gene cluster, a two–step gene deletion procedure was used. Flanking regions from within the *antC* gene of approximately 1.4 kb were amplified using the High-

Fidelity PCR kit (Roche). The primers (region 1–upstream and region 2–downstream) were engineered at their 5' ends with restriction sites suitable for cloning into the *Streptomyces*/*E. coli* shuttle vector pKC1139. The primers for the △antC are listed in Table 6.2. The restriction sites that were used for region 1 (R1) were Hind III/XbaI and for region 2 (R2) were XbaI/EcoR1. The PCR products were cloned into pJET1.2/blunt using the Fermentas PCR cloning kit giving pJETA1 and pJETA2 corresponding to the upstream and downstream (R1 and R2) of the *antC* gene respectively. Around 10 clones from each were sequenced to verify the inserts and an error free clone was selected for each. Region 1 was excised from pJETA1 using HindIII and XbaI and cloned into the similarly digested pKC1139 resulting in pKC1139A1. pJETA2 (pJET1.2/blunt containing region 2) was digested with XbaI and BamHI and the region 2 insert was cloned into similarly digested pKC1139A1 resulting in pKC1139A1A2 which contains both the flanking regions.

The pKC1139A1A2 was conjugated to *Streptomyces* sp. SM8 strain. Competent cells of *E. coli* C2925/pUZ8002 were transformed to apramycin resistance with pKC1139A1A2. Strain C2925 was used as it is *dam* and *dcm* methyltransferase deficient as unmethylated DNA was required for efficient conjugation into *Streptomyces* SM8. The helper plasmid pUZ8002 can supply transfer functions to *oriT*–carrying plasmids, such as pKC1139, but is not efficiently transferred itself because of a mutation in its own *oriT* (Paget *et al.*, 1999). The *E. coli* clones of C2925/pUZ8002/pKC1139A1A2 were grown in 10 ml of LB containing 25 µg kanamycin/ml, 25 µg chloramphenicol/ml and 50 µg apramycin/ml overnight at 37°C. The overnight culture was diluted (1:10) in LB containing the aforementioned antibiotics and grown at 37°C to an OD_{600}=0.5. The cells were washed twice with an equal volume of LB to remove antibiotics that might inhibit the growth of *Streptomyces*. The cells were resuspended in 0.1 vol. of LB. Approximately, 10^8 spores of the SM8 strain was added to 500 µl of 2×YT broth (1.6% peptone, 1% yeast extract and 0.5% NaCl) and subjected to heat shock at 50°C for 10 min. 0.5 ml of *E. coli* cells were added to the spores and mixed briefly. The pellet was resuspended and plated on to Mannitol Soya agar (20 g mannitol/l, 20 g soya flour/l and 20 g agar/l) containing 10 mM $MgCl_2$ and 33.g Instant Ocean/l. The plates were incubated at 28°C for 20 h. The plates were overlayered with 1 ml antibiotic solution containing 25 µg nalidixic acid and 100 µg of apramycin using a spreader. These plates were further incubated at 28°C until potential exconjugants were observed. The transconjugants were plated on to SYP–SW media containing 25 µg nalidixic acid/ml and 100 µg apramycin/ml (Kieser *et al.*, 2000a) and incubated at 28°C until exconjugants were observed.

The shuttle vector pKC1139A1A2 has a ts *ori* (pSG5), temperature–sensitive origin of replication. In *Streptomyces* it can replicate only at temperatures between 28°C and 30°C. When potential exconjugants were plated on to SYP–SW containing 100 µg apramycin/ml and incubated at 37°C, the vector stops replicating and apramycin resistance is only maintained when the plasmid is integrated onto the chromosome by homologous recombination. Apramycin resistant recombinant clones were allowed to sporulate on SYP–SW agar at 37°C. Spores were collected and plated out to single colonies at 28°C without selection. Single colonies were

then picked and screened for the loss of apramycin resistance, a phenotype indicating that the plasmid has been lost and potentially deletion of the *antC* gene. Loss of the pKC1139A1A2 plasmid backbone and the mutagenesis of the *antC* in the △antC was confirmed by PCR using the primers listed in Table 6.2.

Table 6.2 : Primers designed for the construction and analysis of the antimycin-knockout strains.

Name of the primers	Primer sequences (5′–3′)
antC 1	FP–TATATAAAGCTTGGACGGCTACAGCTACAAGC
	RP–TATATATCTAGAATGAGGTATGCGGTGTCGTA
antC 2	FP–TATATATCTAGAGAGGTGGTTCGTGGAGGAG
	RP–TATATAGAATTCTGACGATGATGACGTCCTTG
*ant*1 FP	FP–CCCATTCGCTCCTTGAATTA
*ant*3 FP	FP–CCTTCGAACAGCTCGTCCT
*ant*5 RP	RP–GAGTCGACGACGGGAAGAT

6.4 Results

6.4.1 Genome sequencing and analysis

Streptomyces sp. SM8 was isolated from the marine sponge *Haliclona simulans* collected off the west coast of Ireland, and initial chemical screening showed that it displayed both antimicrobial and anti-calcineurin activity (Chapter 5). The genome was sequenced from a fragment library using Roche 454 pyrosequencing at the University of Liverpool resulting in 229,280 reads, giving approximately 13–fold coverage. There are 464 contigs with an average size of 15,411 kb and the N50 value of the genome is ~27 kb (i.e. 50% of the genome is covered by contigs greater than 27 kb). The N90 value is ~8 kb. These reads were quality filtered and assembled using the meta-assembler hosted by CAMERA 2.0 (Sun *et al.*, 2011) and quality filtered resulting in 539 contigs. The contigs were annotated by IMG/ER (Markowitz *et al.*, 2009) using the "Prodigal" pipeline (Hyatt *et al.*, 2010). The annotation process removed a further 5 contigs for quality reasons, leaving 534 contigs and a predicted genome of approximately 7.15 Mb. The genome statistics are represented in Table 6.3.

Table 6.3 : Genome summary of SM8.

The genome statistics are based on those predicted by IMG (`http://img.jgi.doe.gov/`). PKS and NRPS tabulated are predicted by COG.

	Statistics
Genome size	7.15Mb
Contigs	534
GC content	73.32%
Gene count	6722
Coding genes with function prediction	4625
Coding genes connected to KEGG	1351
Coding genes connected to COGs	4618
Coding genes connected to Pfam	4738
Coding genes connected to TIGRfam	1326
Fused protein coding genes	23
Coding genes coding signal peptides	2100
Coding genes coding transmembrane proteins	1517
NRPS (COG 1020)	20
Type I PKS (COG 3321)	28
Type II PKS (COG 0304)	3
Lantibiotic	1
Terpene	2

The SM8 genome appears to contain several regions coding for potential secondary metabolites, including PKS, NRPS (including PKS/NRPS hybrid clusters), terpenes and siderophores. Analysis of the genome through a combination of annotation pipelines including RAMMCAP, RAST and antiSMASH led to the identification of contigs containing these genes. The closest matches in BLAST were used to align multiple SM8 contigs together to determine whether a cluster was fully represented. In particular, *Streptomyces* sp. strain SM8 contains clusters potentially coding for candicidin–, antimycin– and gramicidin–like compounds, with high similarity (97–99% nucleotide match) to similar clusters found in other *Streptomyces*, particularly *Streptomyces* sp. S4 (Seipke *et al.*, 2011b); (Seipke *et al.*, 2011a); *S. albus* J1074 (Unpublished, NCBI accession number ABYC00000000) and *Streptomyces* sp. strain FR–008 (Chen *et al.*, 2003). The contigs that correspond to each of the cluster are represented in Table 6.4.

Table 6.4: List of potential clusters found in SM8 and their closest homologue.

Predicted cluster	Type	Closest Homologue	Contigs	Accession Number
Candicidin	PKS I	*Streptomyces* sp. FR–008	127, 178, 183, 187, 215, 216, 217, 218, 291, 292, 324, 340, 345, 346, 347, 348, 349, 468	AMPN01000122, AMPN01000172, AMPN01000177, AMPN01000181, AMPN01000209, AMPN01000210, AMPN01000211, AMPN01000212, AMPN01000284, AMPN01000285, AMPN01000314, AMPN01000330, AMPN01000335, AMPN01000336, AMPN01000337, AMPN01000338, AMPN01000339, AMPN01000455
Unknown NRPS1	NRPS	*Streptomyces* sp. S4	440, 352, 113	AMPN01000427, AMPN01000342, AMPN01000108
Unknown NRPS2	NRPS	*Streptomyces* sp. S4	223, 151	AMPN01000217, AMPN01000145
Gramicidin	NRPS	*Streptomyces* sp. S4	392, 68, 32, 70, 326, 536	AMPN01000380, AMPN01000067, AMPN01000031, AMPN01000069, AMPN01000316, AMPN01000511
Unknown NRPS3	NRPS	*S. albus* J1094, *Streptomyces* sp. S4	6, 274, 39	AMPN01000006, AMPN01000267, AMPN01000038
Unknown Hybrid1	Hybrid PKS/NRPS	*Streptomyces* sp. S4	180, 271, 424, 406	AMPN01000174, AMPN01000264, AMPN01000411, AMPN01000393
Antimycin	Hybrid PKS/NRPS	*Streptomyces* sp. S4	51, 443, 475, 406	AMPN01000050, AMPN01000430, AMPN01000462, AMPN01000393
Unknown Hybrid2	Hybrid PKS/NRPS	*Streptomyces* sp. S4	206, 195	AMPN01000200, AMPN01000189
Unknown Hybrid3	Hybrid PKS/NRPS	*Streptomyces* sp. S4	111, 252, 253, 255, 256, 327, 376	AMPN01000106, AMPN01000245, AMPN01000246, AMPN01000248, AMPN01000249, AMPN01000317, AMPN01000364
Unknown PKS II	PKS II	*S. aureofaciens*	21	AMPN01000020
Unknown siderophore1	Siderophore	*Streptomyces* sp. S4	99, 229	AMPN01000095, AMPN01000222
Unknown Terpene	Terpene	*S. albus* J1094	399	AMPN01000386
Lantibiotic	Ribosomal peptide	*S. albus* J1094	266, 169, 203	AMPN01000259, AMPN01000163, AMPN01000197
Unknown siderophore2	Siderophore	*Streptomyces* sp. S4	112, 40	AMPN01000107, AMPN01000039
Unknown Terpene	Terpene	*Streptomyces* sp. S4	269	AMPN01000262

The DNA sequences of the contigs were analysed in BLAST and the closest homologues were aligned to the contigs using CodonCode aligner. Genome analysis of SM8 identified one Type I PKS that was predicted to be part of gene cluster for the biosynthesis of candicidin. The candicidin gene cluster in SM8 spans around 18 contigs. Four NRPS gene clusters were identified, one of which was predicted to be involved in the biosynthesis of gramicidin and contained up of 6 contigs. The metabolites produced by the other NRPS gene clusters were unknown and all of the clusters identified were 98% identical to the clusters previously identified in *Streptomyces* S4 strain. An entire antimycin biosynthesis gene cluster, synthesised by a hybrid PKS/NRPS pathway, was identified in the SM8 genome and comprised four contigs. It is interesting that the unknown hybrid1 cluster is located immediately adjacent (70 bp apart) to the antimycin biosynthesis gene cluster, with genes predicted to be involved in the biosynthesis of some other metabolite. The metabolites produced by the other clusters are unknown. Two siderophore biosynthesis clusters identified are predicted to be synthesised by NIS. One lantibiotic biosynthesis cluster was also identified in SM8. The secondary metabolism gene clusters were identified by antiSMASH (Medema *et al.*, 2011).

6.4.2 Transcriptional analyses

The first nine clusters tabulated in Table 6.4 were selected for transcriptional analyses as they were predicted to be involved in the production of antibiotic–like compounds. These clusters were analysed by antiSMASH, PKS/ NRPS analyser and SBSPKS and all of the nine clusters were found to encode proteins with domain structures typical of enzymes involved in bioactive secondary metabolite production. In order to analyse the transcription of these gene clusters a single gene, predicted to encode a biosynthetic enzyme, was selected from each cluster. PCR primers were designed for each of these genes using Primer3plus and PCR conditions were optimised using genomic DNA as the template with products validated by sequencing and comparison to the target sequence.

As described in Chapter 5, SM8 was grown in five different growth media to maximise the production of different secondary metabolites (Goodfellow & Fiedler, 2010). It was observed that growth in the oatmeal media with the artificial seawater produced 3 fold more of the activity than the SYP–SW when tested in the NCCLS assay for anti–fungal activity against *C. albicans* (as discussed in Chapter 5, Section 5.3.4.3). Hence the strain was grown in both SYP–SW and OM–SW to compare the expression of various clusters. RNA was isolated and cDNA was synthesised as described in Section 6.3.6. This was followed by PCR using primers in table 6.1 and agarose gel electrophoresis to provide semi–quantitative analysis of transcription of each of the gene clusters. Transcriptional analyses showed that no transcript was detected for the putative candicidin biosynthetic genes, unknown NRPS1, unknown NRPS2 and unknown Hybrid1 under any of the conditions tested. The analysis also revealed expression of the unknown NRPS3 cluster when SM8 was grown in OM but not in SYP. The unknown NRPS3 was also found to be present in scaffold 5 of the *Streptomyces* S4 genome but was not annotated

by the group (Seipke *et al.*, 2011a). Our study also identified active transcripts for potential antimycin, gramicidin and two unknown hybrid clusters (2 and 3) (Figure 6.1).

In an attempt to correlate bioactivity with gene expression data, the supernatants from both media conditions were collected on days 2, 4, 6, 8, 10 and 12 and analysed for antimicrobial activities (Figure 6.2) using the NCCLS assay described in Chapter 5, Section 2.4. It was observed that extracts from day 6 showed the highest anti–fungal activity against *C. albicans* in both OM and SYP media but the anti–bacterial activity against *B. subtilis* was highest in day 6 in OM with no anti–bacterial activity being observed after day 4 in SYP. Another interesting observation was that the relative units varied greatly. Both anti–fungal and anti–bacterial activities were almost 4–fold higher on day 6 in OM medium compared to that in SYP. This shows that both the growth medium and timing play an important role in the production of antibiotics.

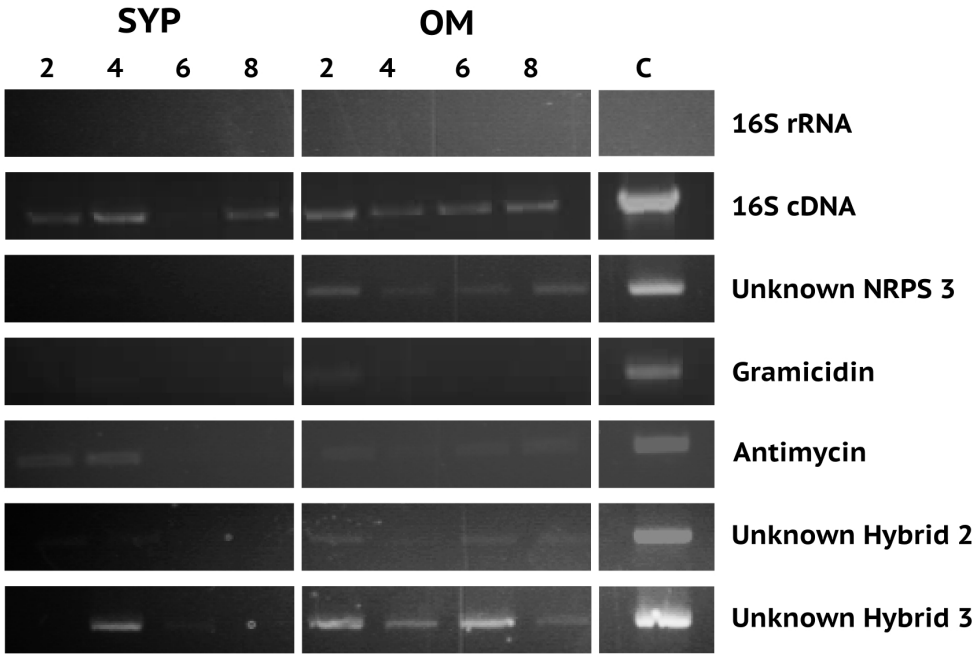

Figure 6.1 : Transcriptional analyses of the secondary metabolism gene clusters identified in SM8.

RT-PCR analyses were performed on the RNA samples collected on day 2, 4, 6 and 8. cDNA was not observed for day 10 and day 12 as the quantity of the initial RNA was low. All RNA preparations were analysed using primers for the 16S rRNA gene to confirm that there was no contamination of genomic DNA. 16S PCR of the cDNA served as a positive control for the RT reaction. Clear expression was observed in five of the nine clusters that were identified in SM8. The difference in the expression levels from the two different media (SYP and OM) is clearly observed. Lane C is a positive control (genomic DNA).

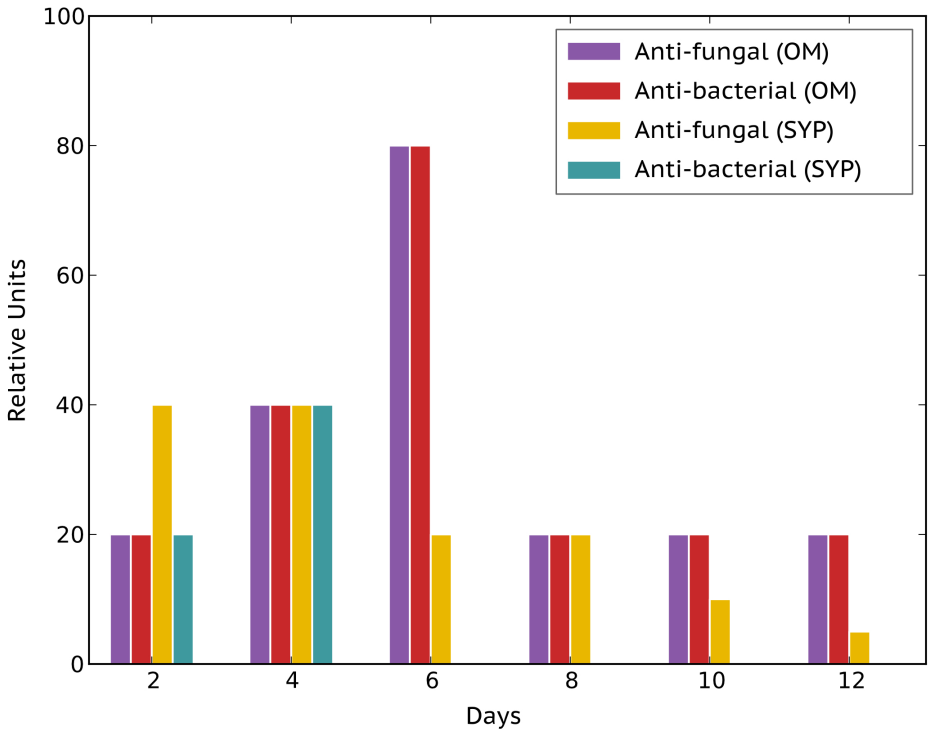

Figure 6.2 : Antimicrobial activities observed in OM and SYP media.

The relative units were expressed as 5 units/well of activity in the NCCLS assay. Hence on day 2 in OM, the activity was seen in four wells and so it was calculated to be 20. The same is true for the SYP medium. In OM, both the anti-fungal and the anti-bacterial activities that were observed were the same. The activities were highest on day 6 and the activity gradually decreased thereafter. In SYP, the anti–bacterial activity was observed only on days 2 and 4 whereas the anti–fungal activity was the same on both days 2 and 4 and then it gradually decreased.

6.4.3 Correlation of genomics, bioactivities and chemical analyses

Transcriptional analyses showed clear expression of the antimycin gene cluster and the chemical analyses (Chapter 5, Section 5.4.2.3) also showed antimycin to be present in the anti–fungal fractions suggesting that at least part of the anti-fungal activity could be due to this known anti–fungal compound. It can be hypothesised that the metabolites produced by the other clusters (NRPS3 and Hybrid 2, 3) could also be contributing to the anti–fungal activity based on the RNA expression levels in OM shown in Figure 6.1. With the anti–bacterial activity, it appears that the activity is not due to the gramicidin cluster as no expression of the gramicidin genes was observed on day 6 in any of the conditions tested. Hence it can be hypothesised that the anti–bacterial activity detected in OM could be due to the product(s) of one of the other clusters (Unknown NRPS3, Unknown Hybrid3 and antimycin) expressed by SM8.

It was observed in Chapter 5, Section 5.4.2.3, that anti–calcineurin activity was exhibited by a butenolide compound. Butenolides belong to a class of lactones that include a family of α, β and γ unsaturated lactones. Studies have shown that the butenolides are signaling molecules that can control pathogenicity and antibiotic production in *Streptomyces*. It has been seen that these signaling molecules are autoregulators and the most characterised γ–butyrolactone autoregulators are A–factor, methylomycin furan (MMF) and avenolide in the avermectin biosynthetic pathway (Kitani *et al.*, 2011). Recently, SRB1 and SRB2 have also been identified as signaling molecules for the production of lankacidin and lankamycin in *S. rochei* (Arakawa *et al.*, 2012). In most of the *Streptomyces* species studied to date, the genes involved in autoregulators biosynthesis and that encode the receptor proteins are often regulated in the same cluster. Hence we searched for homologs of the γ–butyrolactone autoregulator receptor protein consisting of *avaR1*, *avaR2*, *avaR3* and two genes *aco* and *cyp17* in the SM8 genome using BLASTx. However no significant matches were found in the genome. It should be noted that the furanones exhibiting anti–calcineurin activity in SM8 belong to a family ofα, β –unsaturated lactones. There is no known biosynthesis pathway for this family of butenolides and without further experimental data it is not possible to identify genes that may be involved in the biosynthetic pathway of this family of butenolide compounds.

6.4.4 Analysis of a novel hybrid cluster

The unknown hybrid cluster3 identified in SM8 was found to be 98% identical to a cluster in *Streptomyces* sp. S4 (Seipke *et al.*, 2011a). Due to high and consistent gene-expression levels and the activities that were observed in Figure 6.1 and 6.2, the cluster was selected for more detailed bioinformatic analysis. The contigs were aligned with the homologous cluster from *Streptomyces* sp. S4 using the CodonCode Aligner. Predicted gaps between the contigs ranged from approximately 500 to 700 bp. To confirm and close these gaps primer pairs were designed to amplify the predicted gaps from genomic DNA. All pairs used resulted in products of the predicted size and the PCR products were sent for DNA sequencing analysis. The sequences were then manually edited in the GeneQuest software (`http://www.dnastar.com/t-sub-products-lasergene-genequest.aspx`) and all gaps in this cluster were closed resulting in a predicted size of ~50kb. The ORFs were predicted by GeneMark (Besemer & Borodovsky, 1999) and then verified by BLASTp. The start and stop codons for each of the genes were manually verified in Seqbuilder (`http://www.dnastar.com/t-sub-products-lasergene-seqbuilder.aspx`). The predicted proteins were analysed by BLASTp and the secondary metabolism genes by antiSMASH. The PKS/ NRPS domains were also analysed by PKS/ NRPS analyser (Bachmann & Ravel, 2009). The predicted genes with the predicted PKS/ NRPS domains are shown in Figure 6.3, Table 6.5.

Table 6.5 : Predicted genes in the unknown hybrid cluster3 of SM8.

ORF	Predicted function	Start	Stop
1	Subtilisin	32	907
2	Predicted protein/GAF domain	891	1478
3	Integral membrane protein	2083	1553
4	Short-chain dehydrogenase	2245	3042
5	Conserved hypothetical protein	3138	3452
6	Hypothetical protein	3553	4428
7	Monoxygenase FAD binding protein	4969	6210
8	Hypothetical protein	6543	7703
9	NRPS/PKS	7706	16948
10	PKS	16945	25875
11	PKS	25914	32039
12	DNA alkylation repair enzyme	32032	33153
13	PKS	33150	36905
14	Predicted protein	36971	37279
15	Histone deacylase	37276	38424
16	Thioesterase	38417	39249
17	Major facilitator protein	39256	40812
18	Glyoxylase/Bleomycin family protein	41325	40897
19	Glyoxylase family protein	41522	41896
20	Hypothetical protein	41943	42530
21	TetR family transcriptional regulator	43190	42540
22	TetR family transcriptional regulator	43504	44361
23	Conserved hypothetical protein	44431	45324
24	3–hydroxyacyl–CoA dehydrogenase	45351	46382
25	4–hydroxybenzoylCoA–Thioesterase	46379	46858
26	Hypothetical protein	46986	48026
27	Transposase	49493	48879
28	Transposase	49627	49433
29	Conserved hypothetical protein	49779	50246

The hybrid cluster in SM8 appears to contain three PKS genes (ORF 10, 11 and 13) and one hybrid PKS/NRPS gene (ORF 9) which are predicted to be involved in the biosynthesis of the metabolite. The presence of a DNA alkylation repair enzyme encoded by ORF 12 could be an indication of a potential resistance mechanism implying that the active compound could be DNA alkylating agent. ORF 17 is typical of small molecule transporters often involved in the export of antibiotic compounds. ORF 21 and 22 appear to encode regulatory genes that one would predict to be involved in the regulation of antibiotic production by SM8. Studies have

identified a TetR family transcriptional regulatory gene as a global antibiotic regulatory gene in a DNA microarray analysis from interspecies *Streptomyces* (Lee *et al.*, 2010). ORF 27 and 28 are likely pseudogenes. ORF 24 and 25 could be involved in the production of precursor compounds for use by the NRPS module. Further studies are however needed to understand the role of each of the genes involved in the cluster.

From a more detailed analyses of the NRPS and PKS modules, it is apparent that the cluster lacks the AT domain at two PKS modules based on the PKS/NRPS analyser bioinformatic tools used (Medema *et al.*, 2011); (O. Bachmann & Ravel, 2009). PKS lacking integrated AT domains typically have a discrete AT that selects extender units. So far, only four types of discrete ATs have been identified: AT (a free–standing AT), AT–AT (tandem), AT–ER (AT fused with ER), AT–AT–ER (tandem AT integrated with ER) (Musiol & Weber, 2012); (Piel, 2010). But this cluster lacks all of the above mentioned architectures with the only ATs present being at the C–terminus of predicted PKS proteins. Analysis of the genome of SM8 also did not reveal any likely discrete (or *trans*) AT domains. Phylogenetic analysis of the AT domains (Figure 6.4) was performed to identify the signature of the AT domains present within the cluster (Musiol & Weber, 2012) and it was observed that the AT domain in ORF 9 and 11 were not clearly within either the *cis* or *trans* AT clade, forming a separate clade. As this cluster lacks an identifiable discrete AT it may be that the ATs present on ORFs 9 and 11 may act as both *trans* and *cis* ATs.

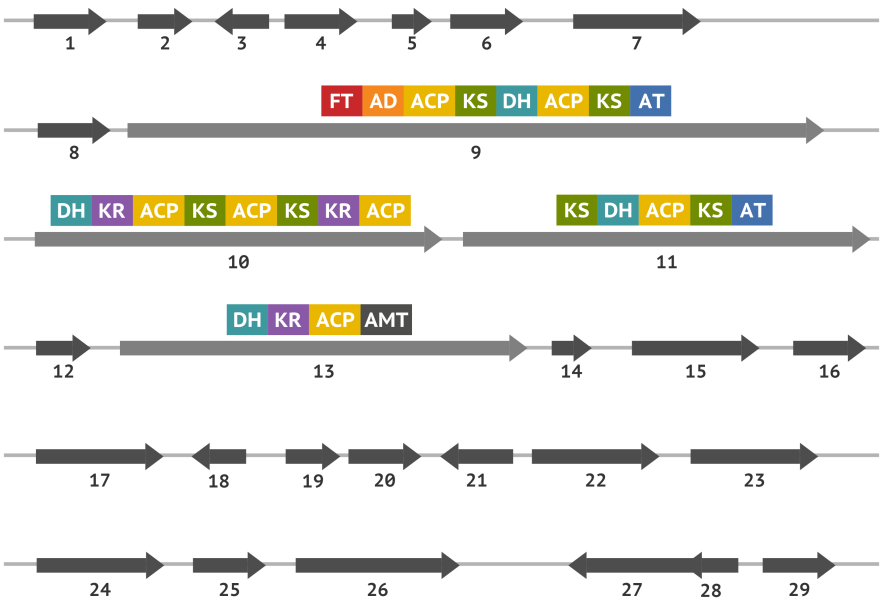

Figure 6.3 : Gene prediction of the Unknown Hybrid cluster3.

The PKS/NRPS domains of the secondary metabolite genes are labelled above their respective PKS/-NRPS genes. ORF are designated as numbers and the PKS and the hybrid NRPS/PKS are denoted by grey colour.

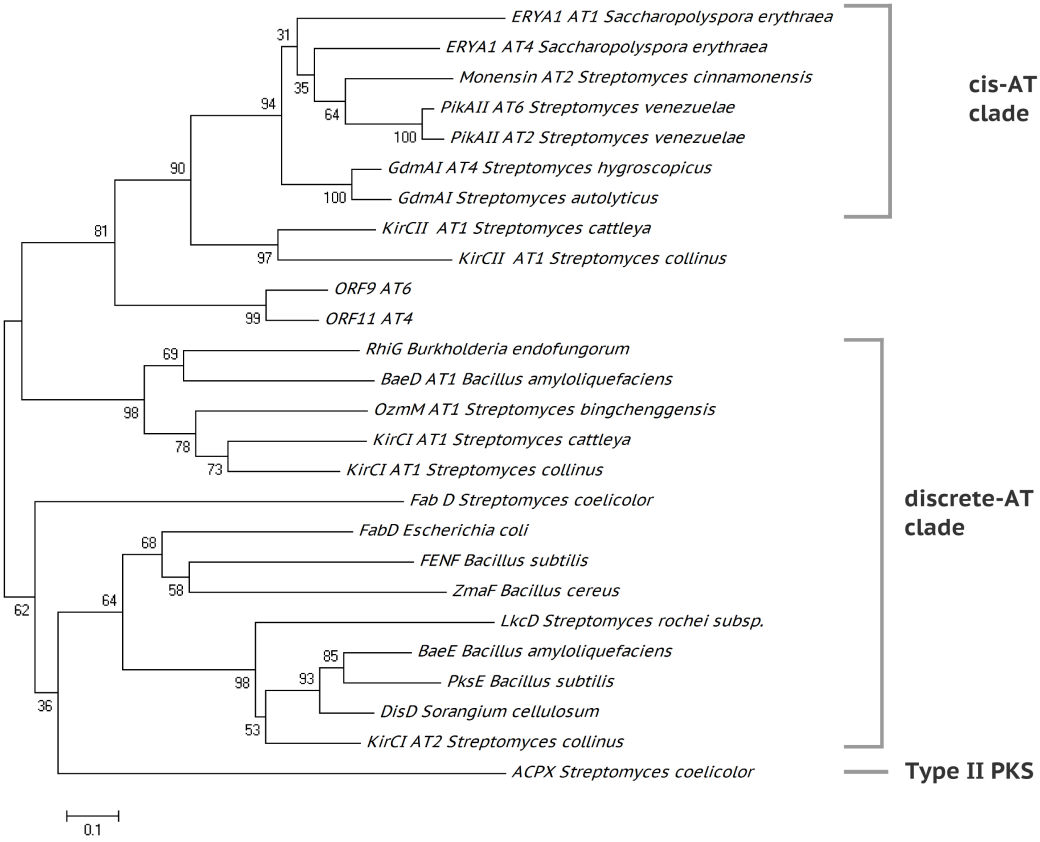

Figure 6.4 : Phylogenetic analysis of AT domains associated with the polyketide biosynthesis.

The AT domain of both Kirramycin and the unknown hybrid cluster3 does not belong either to *cis* or *trans* AT clade. The tree was calculated on a dataset representing AT domains associated with both cis–AT and trans–AT. It is seen clearly that the AT from ORF 9 and ORF 11 can either be in *cis* or *trans* AT clade. All ATs are malonyl–CoA specific exceptions are▲ are methyl malonylCoA specific and ♦ ethyl–malonylCoA specific. The evolutionary history was inferred using the Neighbor–Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 245 positions in the final dataset (Zuckerkandl & Pauling, 1965). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

A prediction of the secondary metabolite produced by the hybrid cluster was performed based on the ORF prediction and domain structure (Figure 6.5). The adenylation unit in module 1 is predicted to adenylate 3–hydroxy–4–methyl–phenylalanine based upon PKS/NRPS analyser. An unusual formylation domain in the NRPS is predicted to attach a formyl group to the amine

that has been so far reported only in the synthesis of oxazolomycins (Zhao *et al.*, 2010). The remaining PKS catalysed steps proceed using standard PKS steps. In addition to the unusual arrangement of AT domains, other unusual features of this PKS system are the lack of a KR domain in module 3 making the DH domain redundant and the terminal aminotransferase domain in module 4 for which no clear role has been proposed though it is possible that the biosynthesis is terminated by the addition of an amine to the polyketide.

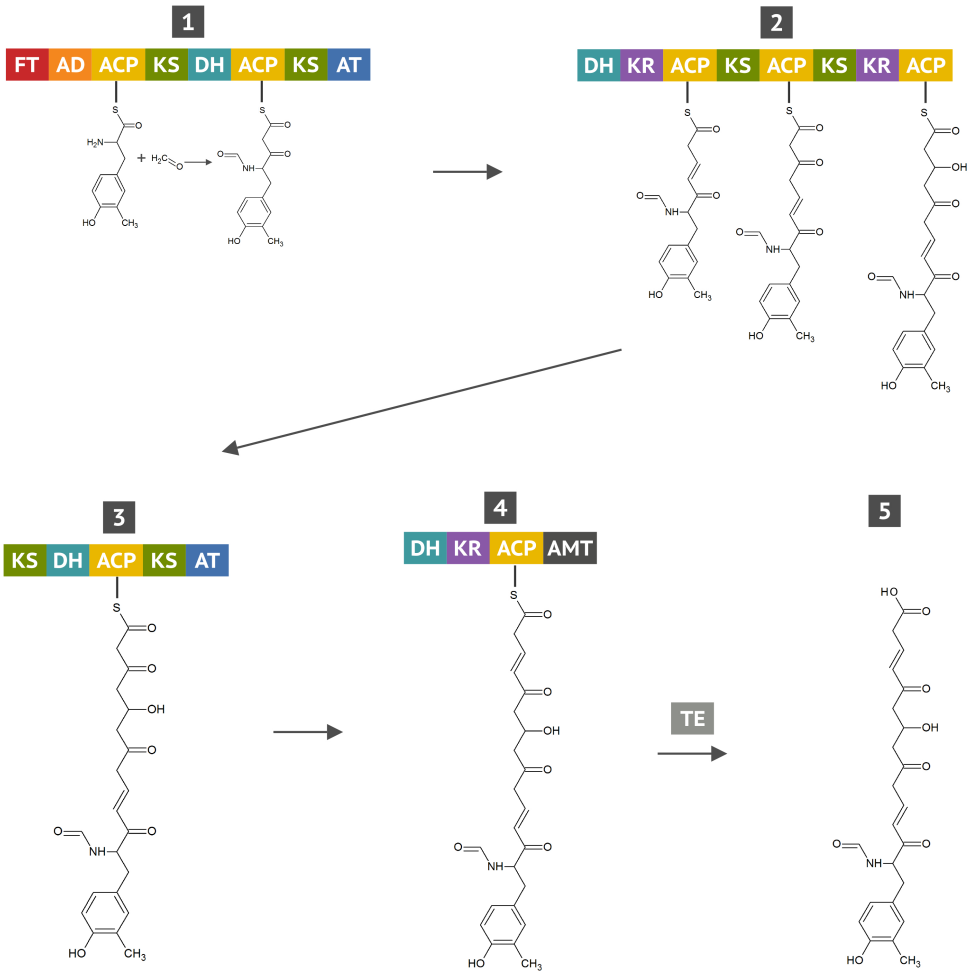

Figure 6.5 : Prediction of the biosynthetic pathway of the Unknown Hybrid cluster3.

Biosynthesis of the metabolite is initiated when the AD (adenylation domain) selects the starter unit, 3–hydroxy 4–methyl phenylalanine and loads on to the ACP. A formyl group in the NRPS module is added to the growing chain which is catalysed by the formyltransferase. Biosynthesis proceeds by the decarboxylation caused by the KS domain. The *cis* AT in module 1 acts in *trans* in module 2 and the elongation process proceeds. The same holds good with the *cis* AT in module 3 acting in *trans* with module 4. Finally, the chain stops when the TE domain is recognised. This then releases the final product as seen in step 5.

6.4.5 Mutagenesis of antimycin gene cluster

The expression of the antimycin cluster in SM8 suggested that the anti–fungal activity could be due to antimycin and the biochemical analyses also showed some antimycin–like compounds in the anti-fungal fraction as discussed in Chapter 5, Section 5.4.2.3. Biosynthetic (Seipke *et al.*, 2011a) and feeding studies (Schoenian *et al.*, 2012) with labelled precursors have proposed an enzymatic pathway for the biosynthesis of antimycins. On this basis we selected the NRPS gene, *antC* for inactivation (Figure 6.6).

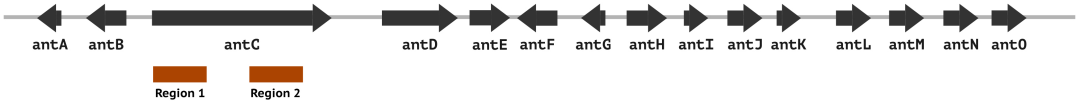

Figure 6.6 : Antimycin gene cluster in *Streptomyces* SM8.

The two regions (1 and 2) of the NRPS gene, *antC* that were cloned to construct the deletion cassette are indicated.

To confirm whether antimycin alone was responsible for the anti–fungal activity, the antimycin gene cluster was disrupted by the deletion of part of the NRPS gene, *antC* (Figure 6.6). The *antC* gene is present in contig 443 and is ~9 kb in SM8. Two regions of *antC* (A1, A2) each ~1.4 kb were PCR amplified and were cloned to an *E. coli*/*Streptomyces* shuttle plasmid (pKC1139). The shuttle vector pKC1139A1A2 containing the *ori-T*, *ts* and ap^R (apramycin resistance gene) was then conjugated to SM8 strain. The apramycin resistant conjugants were selected after incubating the plates at a higher temperature (37°C) and these were called as intermediates (IN). The conjugants were then replica plated on to SYP–SW with and without apramycin. ~190 colonies were screened for apramycin sensitivity when incubated at 37°C. Three putative mutant colonies showed apramycin sensitivity indicating the loss of the plasmid backbone. These mutants were screened using PCR to confirm the deletion in *antC*. Primers were designed for specific regions as seen in Figure 6.7. After PCR screening using primers listed in Table 6.2, one of the strain showed a loss of ~2 kb (using primers 1 and 5) when compared to the wild–type strain indicating that the strain could be a mutant, △antC (Figure 6.8). To verify this, PCR was performed with the other primer pairs for WT, IN and mutants. PCR using primers 3 and 5 showed a 2 kb product in WT and no bands were observed in the mutant. There was no band observed even in the IN. As variable results were observed when the intermediates were subjected to PCR screening, it was confirmed that the strain was a mutant.

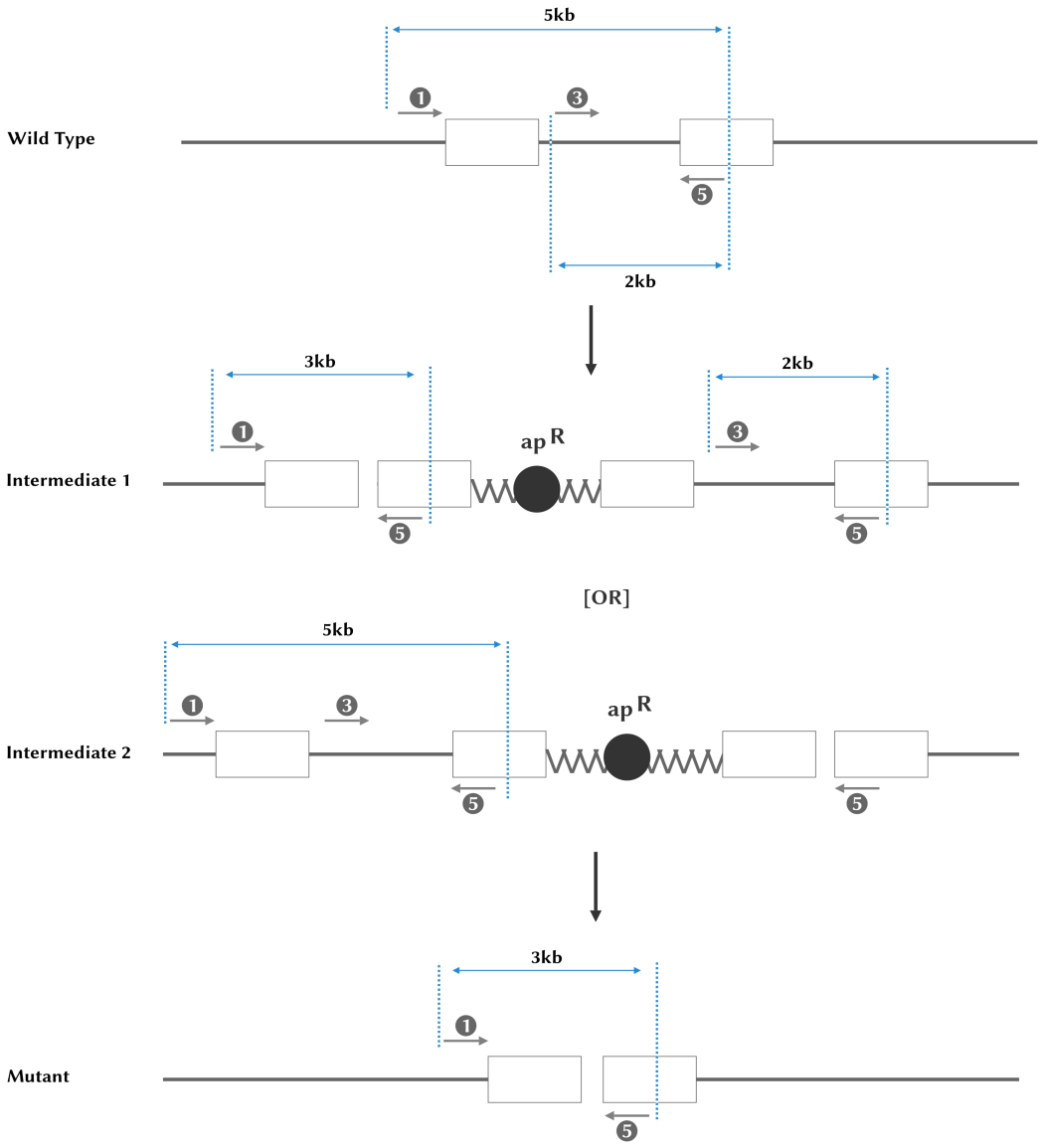

Figure 6.7 : Diagrammatic representation of the wild-type, intermediate and the mutant strain.

The primers used are indicated by the arrows. The black circle shows the apramycin resistance gene present in the plasmid. The first generation of the conjugants were termed as intermediates. All the strains were screened for PCR and the expected PCR product is shown in the figure. PCR screening revealed the loss of the plasmid backbone in the mutant that was not observed in the intermediates and also the mutant was apramycin sensitive. All the strains were screened using PCR primers as mentioned in the figure.

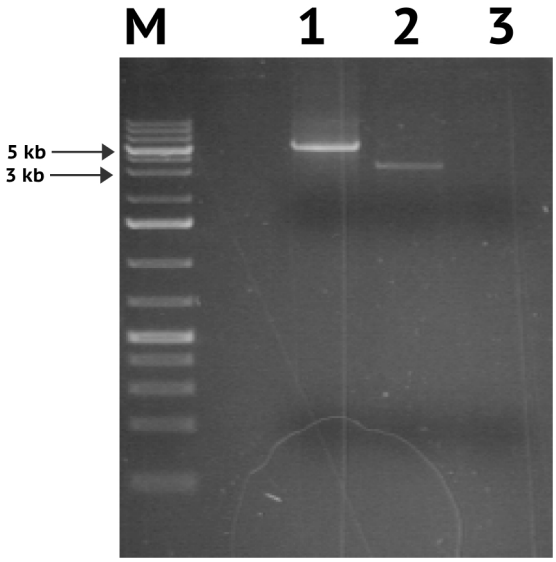

Figure 6.8 : PCR screening of the wild–type and the mutant strain.

PCR screening using primers 1 and 5 showed a product of 5 kb in the WT and a 3 kb in the mutant. The gel shows a loss of approximately 2 kb in lane 2 compared to the wild-type in lane 1. Lane M is the DNA marker of 1 kb plus. Lane 3 is negative control.

The mutant was assessed for anti–fungal activity against *C. albicans* by the NCCLS assay as described in Chapter 5, Section 5.3.4.3. It was observed that the anti–fungal activity was much reduced but not completely abolished. Comparative LC–MS analysis was performed on both wild–type and mutant strains to confirm the absence of antimycin compounds from the mutant strain (Figure 6.9). The antimycin peaks between 20 and 27 min are completely absent in the mutant strain when compared to the wild–type and the standard confirming; that the deletion of the *antC* gene has eliminated antimycin production and; that the anti–fungal activity observed by the mutant strain cannot be due to antimycin. Hence the anti–fungal activity is not solely due to the antimycin compounds but may also be due to some other metabolite present in the mutant strain. The anti–bacterial NCCLS assay was also performed using *B. subtilis* IE32 and *P. aeruginosa* PA01 indicator strains for the extracts. It was observed that the anti–bacterial activity was the same against *B. subtilis* from both the wild–type and the mutant strains but the activity was reduced 3–fold against *P. aeruginosa.* This suggested that antimycin could also be one of the metabolites causing anti–bacterial activity. To confirm this antimycin standards were tested for anti–bacterial activity against *P. aeruginosa* PA01 in the NCCLS assay. It was observed that the antimycin standard showed anti–bacterial activity with an MIC of 0.05 mg/ml. Several studies have tested the antimycin and several analogues against *P. aeruginosa* but none of them has shown anti–bacterial activity (Han *et al.*, 2012); (Atta & Ahmad, 2009). Thus this is the first report of an anti-bacterial activity by antimycin–like compounds.

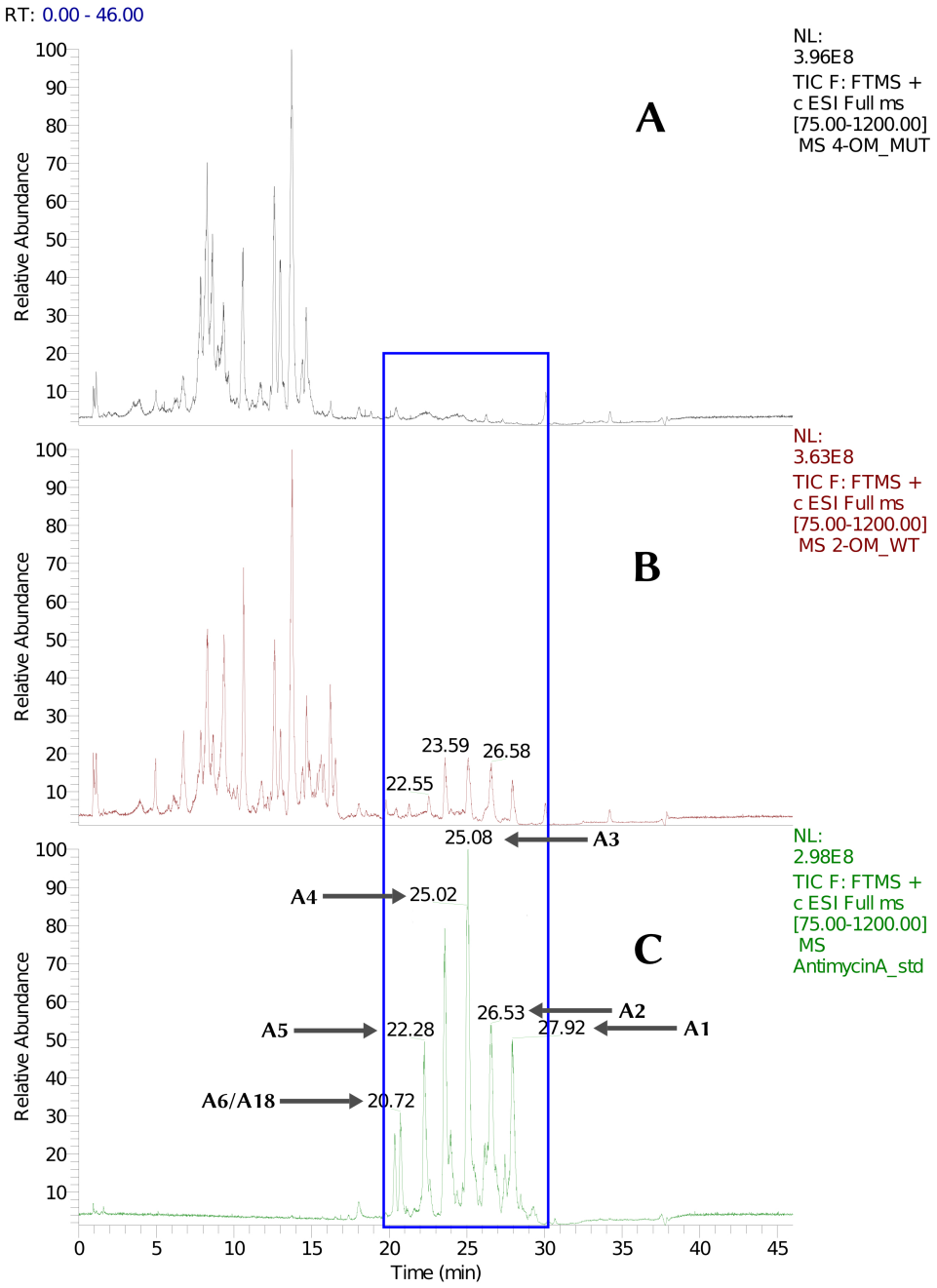

Figure 6.9 : LCMS of the extracts from both wild–type and the mutant strain in OM.

LC-MS analysis was performed by Christina Viegelmann, University of Strathclyde, Scotland.

Panel A–Mutant, B–Wild–type and C–Antimycin standard. In the mutant in panel A, it is clear that the peaks between 22 and 27 min corresponding to antimycins (A1-A4) as observed in both the WT (panel B) and standard (panel C) are missing indicating that the antimycin is completely absent in the mutant. The antimycin peaks (A1, A2, A3 and A4) are indicated in the standard (panel C).

6.5 Discussion

Genome mining approaches applied to *Streptomyces* have in the past revealed an array of novel natural products exhibiting anti–bacterial, anti–fungal and also new terpene classes (Corre *et al.*, 2008); (Chou *et al.*, 2010); (Seo *et al.*, 2011); (Nakano *et al.*, 2011). Genomic analysis of SM8 has revealed the presence of several secondary metabolism gene clusters, some of which are for known metabolites such as antimycin and gramicidin but has also shown clusters whose functions are as yet unknown. Most of the clusters that were identified were similar to clusters of unknown function from *Streptomyces* sp. S4. Transcriptional analyses revealed that several of these clusters of unknown function were being actively expressed (i.e. not strictly silent or cryptic). The metabolites of these unknown clusters can in some cases be predicted after closing the gaps between the contigs as has been described for one of the clusters in SM8.

Two generic approaches that can be employed for determining the function and products of the other clusters are either gene knockout based approaches or approaches involving heterologous gene expression. In SM8, we have used a gene knockout approach to target the antimycin cluster and have demonstrated that the observed anti–fungal activity is likely being contributed not only by antimycin but also by some other metabolites. Hence the same strategy could be employed to allow the identification of metabolites being produced by cryptic clusters and comparative metabolic profiling by LCMS could be used to deduce the metabolites. Such an approach has previously been used by researchers to show the involvement of a novel Type III PKS in the assembly of germicidin by *S. coelicolor*. Deletion of one of the PKS genes from *S. coelicolor* M145 and comparative LCMS profiling showed that the mutant strain failed to produce germicidin (Song *et al.*, 2006). The heterologous expression approach involves the cloning of an entire cluster in a BAC or cosmid vector and the introduction of this cluster into an appropriate heterologous host. LCMS analysis of extracts can then lead to the identification of new metabolites that are most likely the products of the heterologously expressed biosynthetic pathways. This approach has previously been successfully employed to study the production of the antibacterial compound CBS40 (Hornung *et al.*, 2007).

Some additional strategies that can be employed to uncover silent or cryptic biosynthesis clusters involve the so called "genomisotopic approach" and "*in vitro* reconstitution approach". The isotopic approach involves the feeding of the organism with isotope–labelled precursors and screening extracts using 2D NMR experiments. The labelled metabolites can then be purified and further characterised. A novel macrocyclic lipopeptide has been identified in *P. fluorescens* Pf-5 using this approach (Gross *et al.*, 2007). The *in vitro* approach involves incubating substrates with the recombinant purified enzyme and then determining the structures of the resulting products. A novel terpene, epi–isozizaene has been determined by using this strategy (Lin *et al.*, 2006). Both approaches have limitations however, the first approach being somewhat limited due to difficulties involved with high throughput NMR, whereas the second approach requires cloning, expression and purification and may only be useful for simple one enzyme systems. Hence more generic approaches should be employed for identifying the

metabolites produced by the cryptic clusters in SM8.

A more recent generic approach includes the overexpression of the regulatory genes. One study has used constitutive overexpression of a LAL (Large ATP binding of the LuxR) regulator that triggered the expression of a Type I PKS gene cluster in *S. ambofaciens* that led to the identification of four 51–membered glycosylated macrolides, subsequently named stambomycins A–D as metabolic products of the gene cluster with anti-tumor activity. This study represents the largest macrolides ever to be isolated from an actinomycete (Laureti *et al.*, 2011). Another group has used a deregulation approach where they revealed *S. ambofaciens* to be a producer of kanamycin biosynthesis which was known to produce an anti-bacterial compound. This was achieved by deleting the late regulator AlpW and metabolic profiling concluded that the regulation was by a key late repressor of the cellular control in kinamycin biosynthesis (Bunet *et al.*, 2011). In addition various factors such as the size of the gene cluster, the expression of the cluster in laboratory cultures and the accuracy of the products deduced by the bioinformatics analyses also need to be considered when choosing the best strategy to identify the metabolites produced by the cryptic clusters.

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Chapter 7

Discussion

Marine Biotechnology is the application of biological knowledge together with cutting–edge technologies to help develop products and other benefits for humans from various marine sources. The market for marine-related applications is still at its early stages and currently accounts for only a relatively small percentage of the overall economy in the biotechnology market. According to a recent report by Global Industry Analysts, Inc., (`http://www.strategyr.com/`), the global marine biotechnology market is estimated to reach US$4.1 billion in 2015. Marine based biosources have the potential to make a significant impact on the overall socio-economic status worldwide. Hence many countries have relatively recently initiated marine biotechnology programmes to explore and ultimately exploit their marine resources in a sustainable manner. Ireland for example initiated a Marine Biotechnology programme in 2008 to develop innovative research by employing Ireland's extensive marine biological resources.

Ireland's seas are potentially a major source of organisms with biotechnology potential and given that they contain ~90 million hectares of water, the biological diversity likely to be present within Irish water is quite high. Marine sponges which have been harvested from Irish waters form the focus of this study, whereby the microbial diversity has been assessed together with attempts to explore the chemical diversity of these sponges using both culture dependent and independent approaches.

Marine sponges are ancient metazoans that play a vital role in marine ecology. The microbe-sponge symbiotic biology is as yet not well understood. Recent studies have however identified functional genomic signatures such as protein-protein interactions through ankyrin and tetratricopeptide repeat proteins that begin to explain possible mechanisms by which sponges may be able to discriminate between bacteria as a potential food source and bacterial endosymbionts (Thomas *et al.*, 2010).

7.1 Bacterial Diversity Studies

Bacteria associated with marine sponges have previously been shown to be a rich source of a diverse range of chemical classes with bioactive properties including amino acid derivatives, aromatic compounds, alcohols, fatty acids, lactones, polyacetylenes, polyketides, peptides, quinones, quinolones, sphingolipids, sterols, terpenoids and terpenes (Cragg *et al.*, 2009); (Sashidhara *et al.*, 2009); (Leal *et al.*, 2012).

Initial experiments were focused on assessing the bacterial diversity and exploiting the potential of two Irish coastal marine sponges, *A. fucorum* and *E. major* as potential sources of novel chemicals with bioactive properties. Proteobacteria were identified as the dominant group which is similar to reports from other culture-dependent studies in sponges such as *Haliclona simulans* (Kennedy *et al.*, 2008); (Jiang *et al.*, 2007). Culture–dependent studies on the sponge *Ircinia strobilina* have revealed the dominance of both α and β-Proteobacteria, with an increase in the levels of Bacteriodetes being observed when the sponge is cultivated in an aquaculture system (Mohamed *et al.*, 2008). Another study has reported the dominance of γ-proteobacteria and Firmicutes in the marine sponge, *Gelliodes carnosa*, collected from the waters of South China (Li *et al.*, 2011). Another interesting finding from these studies is the low number of Actinobacteria isolated from both *Ircinia strobilina* and *Gelliodes carnosa* in comparison with other studies (Kennedy *et al.*, 2009); although it should be noted that the targeted isolation of Actinobacteria was not carried out here, as in many of the other studies. For example; Xi and his co–workers recovered 90% of actinobacteria isolates from the sponges collected from the South China Sea and the Yellow Sea. They also reported that water agar (M5) medium was the best isolation media in terms of both selectivity and yield (Xi *et al.*, 2012). One other study used culture-dependent approaches to specifically screen for Gram–positive bacteria associated with Antarctic sponges. They used 10 different media and recovered 52% of isolates belonging to Actinobacteria (Xin *et al.*, 2011). The study herein also identified eight *Pseudovibrio* isolates as producers of antibiotics. These findings are in keeping with other studies which have identified members of the *Pseudovibrio* genera are potentially important future targets for the isolation and identification of novel bioactive molecules (O'Halloran *et al.*, 2011); (Santos *et al.*, 2010); (Kennedy *et al.*, 2009). Thus a target-based approach to specifically cultivate fast-growing *Pseudovibrio sp.* may prove useful for the identification of new metabolites.

The diversity studies resulted in the identification of four potential novel species from both sponges when the 16S rDNA sequences were analysed with BLAST. It was observed that the sequences were only 97% or less identical to already known species. Hence phenotypic, biochemical and phylogenetic studies were subsequently employed on one of these species which identified that the novel species belongs to the genus *Aquimarina* isolated from the sponge *Amphilectus fucorum*. The major fatty acid present in this type strain is iso 3-OH $C_{17:0}$ (38.4%) and the percentage of this fatty acid is the highest so far identified in any *Aquimarina* type strain. This together with the presence of other fatty acids which were present at levels

greater than 10% such as iso-$C_{15:0}$ (12.2%), iso 3-OH $C_{15:0}$ (10.1%) and iso-$C_{17:1}$ ω9c (11.8%) makes the strain a novel species which has now been described as *Aquimarina amphilectae* sp. nov. Accessing the bacterial diversity of these marine sponges has therefore provided us with insights into some novel species, and has also provided further clues whereby we can learn how best to cultivate novel bacteria from these particular sponges.

Next-generation sequencing techniques can also provide valuable insights into the microbial populations in marine sponges, with pyrosequencing techniques revealing both sponge-specific novel genera and species. A recent study which compared the use of both culture-dependent and culture-independent approaches to assess the microbial populations in two Irish marine sponges namely *Raspailia ramosa* and *Stelligera stuposa* reported that while only four bacterial phyla could be recovered through culture-dependent strategy; ten phyla could be identified through culture-independent approaches by targeting the V1-V3 region of the 16S rRNA of the sponge metagenomic DNA (Jackson *et al.*, 2012). Another study involving the sponge *Axinella corrugata* has also used pyrosequencing technology to identify novel taxa, with 24% of approximately 65,550 rRNA sequences not matching any bacteria at the class level indicating they could represent novel taxa (White *et al.*, 2012). Another study has also shown how pyrosequencing can be applied to identify sponge specific bacterial communities (Schmitt *et al.*, 2011).

7.2 Calcineurin assay

Work presented in Chapter 4 focussed on the identification of novel inhibitors targeting signalling pathways in yeast using a high-throughput reporter-gene based assay. The assay developed was a microtitre plate assay based on a yeast reporter strain, *S. cerevisiae*. A plasmid pMRK212 carrying a *CRZ1::lacZ* fusion was transformed into the yeast. For the assay, the transformed strain was grown overnight and the calcineurin pathway was initiated by an alkaline stress after the cells were incubated with the test extracts. The cells were lysed followed by the addition of ONPG. The reaction was stopped by the addition of sodium carbonate following development of a yellow colour in the positive control. An absence of the yellow colour indicates that it could be a possible inhibitor. The cells were then measured in the plate reader. Hence a simple, sensitive, reproducible and rapid assay system was developed which can facilitate the screening of more than 1000 compounds in a day. The assay was validated using a reporter-gene *GFP::CRZ1* fusion to demonstrate the localization of the reporter protein. The construct demonstrated the sub–cellular localization of the Crz1p protein and visualized the *GFP::Crz1p* fusion protein in yeast cells by fluorescence microscopy (Polizotto & Cyert, 2001). A pilot screen of 81 bacterial extracts revealed the presence of a butenolide compound in an extract from *Streptomyces* strain SM8 that demonstrated calcineurin inhibition. Future studies such as the interleukin-2 assay (IL-2) and cytotoxicity analysis would be required to confirm the efficacy of the compound. As calcineurin activates the T cells of the immune system, drugs which act as calcineurin inhibitors (CNI) are administered to immunosuppressive

patients. Studies have revealed that calcineurin-dependent IL-2 is required for regulatory T-cells (Treg) *in vivo*. Hence modification of the Treg function by immunosuppressant drugs (such as CNI) can be of clinical importance (Zeiser *et al.*, 2006). The mechanism involving the Ca^{2+}/calcineurin pathway is conserved between yeast and mammalian cells (Rusnak & Mertz, 2000). Hence the inhibitors identified in our study should be further tested in mammalian system and could thus be the subject of further characterisation.

Following screening of a *Haliclona simulans* metagenomic library, eight clones were putatively identified which appeared to display calcineurin inhibition in the aforementioned newly developed assay system. Following sequencing of one of the clones–HS02P13, it appears likely that it may encode a gene encoding an exchanger protein. The clone when analysed in BLASTx displayed 29% identity to a sodium-calcium exchanger protein. The BLAST search did not show similarities to any of the known calcineurin inhibitor genes such as MCIP 1 and MCIP 2 that have shown calcineurin inhibition in striated muscles (Yang *et al.*, 2000). The precise reason behind the observed inhibition in our study is unclear but due to the fact that in a BLASTx search it displayed similarities to a sodium-calcium exchanger protein then it could be removing calcium from the cells which is required for the calcineurin activity. So the inhibition being displayed in the assay may in fact be an artefact of the system. Enzolife Sciences in 2009 have developed a calcium assay kit for microplates called Fluoforte, which detects the mobility of intracellular calcium using a flurogenic binding dye that undergoes an electronic change when calcium is bound which results in a higher magnitude of fluorescence (Martin *et al.*, 2004).

Hence, the HS02P13 clone needs to be further evaluated in a calcium binding assay such as this to confirm that the observed calcineurin inhibition is in fact real. The fact that the *H. simulans* metagenomic is maintained in *E. coli*, may in part be responsible for the lower than expected success rate in isolating clones from the library which are exhibiting calcineurin inhibition. This may result from the potential poor expression of genes derived from the sponge metagenomic DNA in *E. coli*. Hence the use of other expression hosts such as *S. lividans* may result in better expression levels being observed and thus a potential larger number of clones exhibiting calcineurin inhibition. Studies have previously shown *S. lividans* to be a better host when compared to *E. coli* in soil metagenomic libraries so the same may be true for sponge libraries. Scientists from the US constructed a soil metagenomic library in both *E. coli* and *S. lividans* and a functional screening revealed many clones which exhibited hemolytic activity only in *S. lividans* and not in *E. coli* (McMahon *et al.*, 2012). Another expression system that could be potentially useful for cloning larger DNA fragments is the pCCERI that has the property to be conjugated into alternative hosts and also increase the expression levels of the insert in the host (Dunlap *et al.*, 2011).

7.3 Biochemical approach for the identification of bioactives

The identification of novel antimicrobials produced by bacteria associated with the marine sponge, *Haliclona simulans* was the focus of Chapter 5. One of these isolates *Streptomyces* sp. SM8 displayed antimicrobial activity against all the strains tested, (namely *C. albicans*, *C. glabrata*, *S.cerevisiae*, *K. marxianus*, *A. fumigatus*, *B. subtilis* and *P. aeruginosa*). A number of different culture conditions were then employed in an attempt to define growth conditions which would allow maximal production of the antimicrobial compounds. A 3 fold increase in the production of bioactive compounds was observed when a medium comprising of oatmeal and artificial sea water was employed when comparison to the SM8 culture grown in SYP medium and artificial sea water. Culture conditions are known to affect antibiotic production in *Streptomyces* species with numerous examples in the literature including different carbon and nitrogen sources affecting for example production of the nonpolyenic macrolide antibiotic AK-111-81 by *Streptomyces hygroscopicus* 111-81 (Gesheva *et al.*, 2005). Another study has shown mannitol and asparagine as optimal carbon and nitrogen sources for the effective cultivation and the production of the antibiotic production in *Streptomyces* sp. 201 (Thakur *et al.*, 2009). While a recent study has optimized the carbon and nitrogen sources for the production of a protease in the marine-derived *Streptomyces carpaticus* (Haritha *et al.*, 2012).

Other recent studies have reported that optimising culture conditions can lead to an improved production of targeted bioactives, for example; in *Pseudonocardia* sp. VUK-10, an enhanced production of the bioactive compounds was observed when the strain was grown in modified yeast extract-malt extract-dextrose compared to the other media such as the starch-caesin broth and the tyrosine broth (Usha Kiranmayi *et al.*, 2011).

Marine *Streptomyces* are a potential source of novel bioactives exhibiting a broad spectrum of activities. There are many examples in the literature one of which is from an Indian group who identified four isolates of *Streptomyces* sp. that showed antagonistic activity against fish pathogens such as *Vibrio* sp. and *Aeromonas hydrophila*. Thin layer chromatography and spectral analysis showed that the compounds were polyene in nature and could be a promising antibiotic lead (Dharmaraj & Sumantha, 2009). Another study investigated a crude extract of a marine sediment-derived *Streptomyces avidinii* strain SU4 which showed characteristics of a cytotoxic compound. The crude extract of the isolate exhibited a cytotoxic activity of 64.5 µg/ml against a Hep−2 cell line proving that this marine *Streptomyces* isolate is a potential source of a novel anticancer compound (Sudha & Masilamani, 2012). Our study which employed biochemical based approaches resulted in the identification of an array of antimicrobial compounds which included a hydroxylated fatty acid, antimycins, butenolides and an as yet unknown compound of m/z 227. It has been recently shown that fatty acids exhibit anti-bacterial activity (Mohamad *et al.*, 2009); (M. Pereira *et al.*, 2011). In the case of these studies the fatty acids that were observed belong to the unsaturated class of fatty acids. However, in our study, with the fragmentation pattern in NMR, the bioactive compound was identified as a saturated fatty acid. Hence, this is the first report of a saturated fatty acid exhibiting

anti–bacterial activity. The exact mechanism of the antimicrobial action of fatty acids remains unclear; however, the main target appears to be the cell membrane. There has been a suggestion that fatty acids may act by disrupting the electron transport chain or interfere with oxidative phosphorylation, or alternatively may lead to increased fluidity of the membrane ultimately leading to instability and cell lysis (Desbois & Smith, 2010). In fact a recent study has shown that specific fatty acids can inhibit the growth of *Staphylococcus aureus* by disrupting the cytoplasmic membrane which allows metabolites and low molecular weight proteins to leak from the cell (Parsons *et al.*, 2012). A newer class of antimycin compounds have recently been identified from different marine *Streptomyces*. Scientists from China have identified two new antimycin analogues (A19-A20) from the marine actinomycete, *Streptomyces antibioticus* H74–18 (Xu *et al.*, 2011). Another group from China also identified two new antimycin A analogues (B1 and B2) from the fermentation broth of a marine-derived *Streptomyces lusitanus* which showed anti-bacterial activities against *S. aureus* and *L. hongkongensis* (Han *et al.*, 2012). In this current study we identified antimycins that showed anti-fungal activity against *C. albicans*, however, we could not determine the class of antimycins in our marine *Streptomyces* strain SM8 due to an inability to isolate sufficient quantities of the antimycin material for analyses. Hence, an important future goal for this work would be to determine physiological parameters which induce synthesis of these antimycins, such that sufficient qualities of these bioactive molecules could be obtained to allow future structural elucidation studies to be undertaken.

It is widely accepted that currently available extraction techniques for the isolation of bioactives from marine environments need to be improved, as traditional techniques tend to produce low yields, present low selectivity, consume high volumes of solvents and involve long extraction times. Many of the traditional techniques such as soxhlet, solid–liquid extraction (SLE), or liquid-liquid extraction (LLE) are also not automated and hence reproducibility can also sometimes be compromised. Therefore there is an ongoing need to develop and combine cost–effective, selective and environment-friendly techniques as a solution to the aforementioned problems. Recently a number of new techniques have been implemented to overcome some of the above mentioned problems. A group in Spain have recently reported the use of Supercritical fluid extraction (SFE) to obtain and stabilize natural vitamins from red pepper by-products. They used supercritical carbon–dioxide and demonstrated that this method increased the yield of Vitamin-E by 97% (Romo-Hualde *et al.*, 2012). Another group from the US have developed a technique called accelerated solvent extraction (ASE®) or Pressurized liquid extraction (PLE) wherein they identified a series of novel sesquiterpenes from the extracts of five marine sponges. This was achieved by comparing the extraction procedures of the conventional Kupchan approach to the ASE and comparing the metabolic profiling using parallel HPLC (Johnson *et al.*, 2010). Scientists from Singapore have used pressurized hot water for solvent extraction in numerous samples involving food analysis, soil sediments and other environmental samples. They reported that the extraction of certain compounds is dependent on pressurized water (PHWE) with different applied temperatures (Teo *et al.*, 2010). Another

study used Ultrasound-assisted extraction (UAE) to extract carnosic acid and rosmarinic acid from plant samples using an ionic liquid based, ultrasound-assisted extraction technique (Zu *et al.*, 2012). While a Microwave–assisted extraction (MAE) followed by HPLC has recently been developed by Italian researchers where they have quantitatively analysed the levels of the mycotoxin, ochratoxin A (OTA) in commercial roasted coffee beans (Graziani *et al.*, 2012). Hence, it appears likely that some of these approaches could be successfully developed and subsequently employed to improve the extraction efficiency of bioactive compounds from marine microorganisms.

It is clear that extraction procedures to be employed for the isolation of bioactive molecules should be selected based on the nature of the compounds to be isolated. Various parameters that can influence the extraction process include temperature, pH and salt. All extracts should be tested using functional assays before they enter the purification stage. Another strategy that has been successfully employed for the identification of novel bioactives which facilitates an increase in the yield of the product/metabolite under investigation is the use of co–culturing (Penttinen *et al.*, 2006); (Hu *et al.*, 2011). One of the best examples of this approach is the improvement in enzyme production when both *A. niger* and *A. oryzae* were co–cultivated with each other and with *M. grisea* or *P. chrysosporium* respectively. An increased enzyme activity was observed in *A. oryzae* for β–glucosidase, α–cellobiohydrolase, β–galactosidase, and laccase in combination with other fungi, in particular with *P. chrysosporium.* Scientists from Korea identified an active anti-fouling compound named diterpene from a marine *Streptomyces* strain PK209 and they reported that the yield of diterpene increased 10.4 fold when the marine strain was co–cultivated with *Alteromonas* sp. KNS-16 (Cho & Kim, 2012). Another study from China achieved an increased degradation of cypermethrin by co–culturing *Bacillus cereus* ZH-3 and *Streptomyces aureus* HP-S-01, reporting that the half-lives in pure cultures were longer than the mixed cultures (Chen *et al.*, 2012). These reports provide examples whereby the overall levels of bioactive material being produced by the microorganism, which continues to be a bottleneck in the identification of the bioactive compounds; may be increased through approaches such as co-culturing techniques, which together with the optimisation of both extraction process and purification strategies may help overcome this important bottleneck. A combination of these refinements could facilitate a more robust pipeline by ultimately helping in the more rapid identification of novel bioactives thereby facilitating with dereplication strategies in the overall biodiscovery process.

7.4 Genome mining studies

Genome sequencing can provide a platform by which it is possible to both detect and potentially predict the nature of the metabolites being produced by any bacterial species. Hence Chapter 6 focussed on the identification of biosynthetic pathways potentially involved in metabolites production in the SM8 strain. Genome sequencing and subsequent genome mining of SM8 revealed nine secondary metabolic clusters potentially involved in antibiotic pro-

duction in the strain. Some of these clusters are likely to be involved in the production of known metabolites such as antimycin and gramicidin whilst some have as yet unknown functions with high similarity (97–99% nucleotide match) to clusters found in other *Streptomyces*, particularly *Streptomyces* sp. S4 (Seipke *et al.*, 2011b); (Seipke *et al.*, 2011a). Transcriptional analysis revealed the expression of gramicidin, antimycin, one unknown NRPS and two hybrid clusters. As mentioned earlier, the strain was grown for 12 days in both SYP and OM containing artificial sea water and the bioactive compound(s) were extracted in an attempt to correlate the observed activities with that of the gene-expression studies. It was found that the anti–fungal activity was highest on day 6 and while reduced thereafter remained constant after day 8 in SM8 cultures grown on OM medium. But in SYP, however the observed activity was similar on days 2 and 4 but decreased gradually thereafter. With respect to anti-bacterial activity, the observed activity was similar to the anti-fungal activity on the OM growth medium but in SYP, activity was only observed only on days 2 and 4; suggesting that OM may be a better growth medium for anti-bacterial metabolite production than SYP.

The biochemical analyses performed in Chapter 5 had shown that antimycins are one of the metabolites involved in anti–fungal activity which was consistent with gene–expression studies. It can be hypothesised that the metabolites produced by some of the other clusters (NRPS3 and Hybrid 2,3) may be contributing to the anti-fungal activity. The other hypothesis is that the anti–bacterial activity could be contributed by the unknown NRPS3, unknown Hybrid3 and antimycin gene clusters expressed by SM8. The genome of SM8 was interrogated to identify biosynthetic pathway that may be involved in the biosynthesis of butenolides. Butenolides are a class that include a family of α, β and γ unsaturated lactones. Recent studies have reported that the most characterised γ-butyrolactone autoregulators are A–factor, methylomycin furan (MMF) and avenolide in the avermectin biosynthetic pathway in *Streptomyces avermitilis* (Kitani *et al.*, 2011). We searched for the homologue of the existing class, the γ-butyrolactone autoregulator receptor protein, consisting of *avaR1*, *avaR2*, *avaR3* and two genes *aco* and *cyp17* in the SM8 genome using BLASTx but no significant matches were obtained. It should be noted that the butenolides found in SM8 appear to belong to a family of α, β–unsaturated lactones for which no biosynthetic pathway have so far been identified. Hence without further experimental data it is not possible to identify genes that may be involved in the biosynthetic pathway of this family of butenolide compounds.

One of the unknown clusters, namely hybrid cluster3 in SM8 that showed high levels of transcription was further analysed in an attempt to predict the metabolite(s) being produced from this cluster. Genomic analysis of *Streptomyces* sp S4 also resulted in the identification of many putative antibiotic biosynthetic pathways (Seipke *et al.*, 2011a). The unknown hybrid cluster3 from SM8 was found to be 98% identical to the *Streptomyces* sp S4, a single ant symbiont that produced multiple anti–fungals. Based on this level of similarity, it can be predicted that the unknown cluster could be involved in the production of a novel antibiotic. After closing gaps in the available SM8 DNA sequence by PCR, it was possible to predict potential secondary metabolites genes using several bioinformatics tools. An unusual AT domain signature was

identified that could be an integrated (*cis*) AT acting as a discrete (*trans*) AT. If this is the case then this would be the first report to date of this type of AT signature as it does not belong to the four types of discrete AT architecture that have been identified to date in bacteria (Musiol & Weber, 2012). As antimycins were identified both in the biochemical and genomic analysis, it was decided to perform a gene knockout of the antimycin cluster in SM8, to determine whether the observed anti–fungal activity was in fact due to the product(s) of this cluster. A subsequent metabolic profile of both the SM8 wild type and antimycin mutant confirmed that the mutant had lost the ability to produce antimycin. Due to the fact that antimycin production had been lost in the mutant and that the observed anti–fungal activity was not completely abolished in the strain, this suggested that some other metabolite(s) may be contributing to the activity. To identify the additional clusters involved in the bioactivities, various approaches such as gene knockout, heterologous gene–expression and overexpression of regulatory genes could be implemented. Examples of where such approaches have been successfully employed include the study demonstrating the repression of antibiotic production and sporulation in *S. coelicolor* when the TetR family transcriptional regulator was overexpressed by a collaborative approach from scientists from the US and South Korea (Lee *et al.*, 2010). Another example includes the enhanced production of daptomycin, an antibiotic, by overexpressing the accessory genes flanking the NRPS genes responsible for daptomycin production in *S. roseosporus* (Yu, 2012). Some of the other strategies which could be employed to identify the metabolites responsible for the observed anti–fungal activity in SM8 include regulating the transcriptional control by various parameters such as carbon and nitrogen source, pH, temperature and also the addition of transcriptional regulators such as (LysR and DeoR family), photoreceptors (REC) and transcriptional regulators with other DNA binding domains (AraC, Spo0A, Fis) (Bromann *et al.*, 2012). Recently a group from Korea has used genome–based functional discovery of cryptic genes using MS–guided bioinformatic analysis of genomic sequences (Park *et al.*, 2012), which could also prove a useful approach with respect to SM8. Thus genome mining has not only helped in the identification of secondary metabolic pathways but has also given us an insight into the overall genetic make-up of the strain which can be helpful for the discovery of cryptic pathways and also the identification of novel compounds/bioactives.

7.5 Conclusions and future prospects

This study has resulted in the development of a high throughput assay which may prove useful in the identification of calcineurin inhibitors and which may find utility in the marine biotechnology research community as a whole. In addition preliminary genome scanning approaches of the marine isolate *Streptomyces* SM8 have identified several potential secondary metabolism gene clusters that could be involved in the synthesis of novel antibiotics. Genome mining has also identified several silent clusters whose expression needs to be up-regulated so that the nature of the metabolites can be identified. Both chemical analysis and genome

analysis which has been employed on *Streptomyces* strain SM8 have proven to be successful in allowing the discovery of a wide array of bioactives which may be potential future biopharmaceutical applications.

In my opinion, the best pipeline for drug discovery when culture–dependent approaches are being employed is that following initial screening of the isolate to determine bioactivity, the genome of the strain should then be sequenced and subsequently analysed. Whole genome analysis based approaches thus allow for a significant improvement in the overall understanding of the biosynthetic pathways in the producing bacterium, thereby facilitating approaches whereby these pathways can be genetically manipulated to allow the subsequent identification of the relevant novel biocompound(s); while also allowing for approaches to be employed to allow for increased levels of production of these compounds in either the host strain or in heterologous host systems.

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